THE FANCONI ANEMIA SIGNALING NETWORK REGULATES THE MITOTIC SPINDLE ASSEMBLY CHECKPOINT

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"I have been crucified with Christ and I no longer live, but Christ lives in me. The life I now live in the body, I live by faith in the Son of God, who loved me and gave Himself for me." ~Galatians 2:20

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Fanconi anemia (FA) is a heterogenous genetic syndrome characterized by progressive bone marrow failure, aneuploidy, and cancer predisposition. It is incompletely understood why FA-deficient cells develop gross aneuploidy leading to cancer. Since the mitotic spindle assembly checkpoint (SAC) prevents aneuploidy by ensuring proper chromosome segregation during mitosis, we hypothesized that the FA signaling network regulates the mitotic SAC.

A genome-wide RNAi screen and studies in primary cells were performed to systematically evaluate SAC activity in FA-deficient cells. In these experiments, taxol was used to activate the mitotic SAC. Following taxol challenge, negative control siRNA-transfected cells appropriately arrested at the SAC. However, knockdown of fourteen FA gene products resulted in a weakened SAC, evidenced by increased formation of multinucleated, aneuploid cells. The screen was independently validated utilizing primary fibroblasts from patients with characterized mutations in twelve different FA genes. When treated with taxol, fibroblasts from healthy controls arrested at the mitotic SAC, while all FA patient fibroblasts tested exhibited weakened SAC activity, evidenced by increased multinucleated cells. Rescue of the SAC was achieved in FANCA patient fibroblasts by genetic correction. Importantly, SAC activity of FANCA was

confirmed in primary CD34+ hematopoietic cells. Furthermore, analysis of untreated primary fibroblasts from FA patients revealed micronuclei and multinuclei, reflecting abnormal chromosome segregation.

Next, microscopy-based studies revealed that many FA proteins localize to the mitotic spindle and centrosomes, and that disruption of the FA pathway results in supernumerary centrosomes, establishing a role for the FA signaling network in centrosome maintenance. A mass spectrometry-based screen quantifying the proteome and phospho-proteome was performed to identify candidates which may functionally interact with FANCA in the regulation of mitosis. Finally, video microscopy-based experiments were performed to further characterize the mitotic defects in FANCA-deficient cells, confirming weakened SAC activity in FANCA-deficient cells and revealing accelerated mitosis and abnormal spindle orientation in the absence of FANCA.

These findings conclusively demonstrate that the FA signaling network regulates the mitotic SAC, providing a mechanistic explanation for the development of aneuploidy and cancer in FA patients. Thus, our study establishes a novel role for the FA signaling network as a guardian of genomic integrity.

D. Wade Clapp, MD, Chair

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LIST OF ABBREVIATIONS

ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
ANOVA	analysis of variance
APC	allophycocyanin
APC/C	anaphase promoting complex/cyclosome
ATM	Ataxia Telangiectasia-mutated
ATR	ATM-Rad3-related
AURKA	Aurora kinase A
BACH1	BRCA1-associated C-terminal helicase (same as FANCJ/BRIP1)
BLM	Bloom syndrome protein (RecQ-family DNA helicase)
BRIP1	BRCA1-interacting protein (same as FANCJ/BACH1)
BM	bone marrow
BMF	bone marrow failure
BRCA1	breast cancer susceptibility 1
BRCA2	breast cancer susceptibility 2 (same as FANCD1)
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
BUB1, -3	budding uninhibited by benzimidazole 1, -3
BUBR1	budding uninhibited by benzimidazole-related 1
C-MAD2	closed MAD2
CC	FA core complex

CCAN	constitutive centromeric-associated network
CD34	cluster of differentiation (designation) 34
CDC	cell division cycle
CDK1	cyclin-dependent kinase 1 (same as CDC2)
cDNA	complementary DNA
CENP_	centromere protein _
CHK1, -2	checkpoint kinase 1, -2
DMEM	Dulbecco’s modified eagle medium
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ERCC4	excision repair cross-complementing 4 (same as FANCQ)
FA	Fanconi anemia
FAAP	Fanconi anemia-associated protein
FANC_	Fanconi anemia, complementation group _
FBS	fetal bovine serum
FLT3	fms-related tyrosine kinase 3
G1 phase	gap (growth) 1 phase
G2 phase	gap (growth) 2 phase
GFP	green fluorescent protein
GVHD	graft-versus-host disease
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
ICL	DNA interstrand crosslink

ID complex	heterodimer of FANCD2 and FANCI
IRES	internal ribosome entry site
KMN	KNL1 protein and Mis12 and Ndc80 protein complexes
M phase	mitotic phase
MAD1, -2	mitotic arrest deficient 1, -2
MCC	mitotic checkpoint complex
MMC	mitomycin C
MDS	myelodysplastic syndrome
NBS1	Nijmegen breakage syndrome 1
NE	nuclear envelope
NEK2	NIMA [never-in-mitosis-gene A]-related kinase 2
NES	nuclear export sequence
NLS	nuclear localization sequence
O-MAD2	open MAD2
PAGE	polyacrylamide gel electrophoresis
PALB2	partner and localizer of BRCA2 (same as FANCN)
PBS	phosphate buffered saline
PE	phycoerythrin
PLK1	polo-like kinase 1
PP2A	protein phosphatase 2A
RAD51C	human homolog C of yeast Rad51 (same as FANCO)
RNAi	ribonucleic acid interference (post-transcriptional gene silencing)
S phase	synthesis phase

SAC	spindle assembly checkpoint
SCC1, -3	sister chromatid cohesion 1, -3
SCC	squamous cell carcinoma(s)
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
SLX4	structure-specific endonuclease subunit SLX4 (same as FANCP)
SMC1, -3	structural maintenance of chromosomes 1, -3
TPR	tetratricopeptide repeat
UCB	umbilical cord blood
WT	wild type
ZW10	Zeste White 10

CHAPTER ONE

INTRODUCTION

Fanconi anemia

Fanconi anemia (FA) is a rare genetic disease that results from inactivating mutations in any one of sixteen FA genes, which are named *FANCA*, *-B*, *-C*, *-D1*, *-D2*, *-E*, *-F*, *-G*, *-I*, *-J*, *-L*, *-M*, *-N*, *-O*, *-P*, and *-Q* (Strathdee, Duncan et al. 1992, Strathdee, Gavish et al. 1992, Lo Ten Foe, Rooimans et al. 1996, de Winter, Waisfisz et al. 1998, de Winter, Leveille et al. 2000, de Winter, Rooimans et al. 2000, Timmers, Taniguchi et al. 2001, Howlett, Taniguchi et al. 2002, Meetei, de Winter et al. 2003, Meetei, Levitus et al. 2004, Levran, Attwooll et al. 2005, Meetei, Medhurst et al. 2005, Rahman, Seal et al. 2007, Reid, Schindler et al. 2007, Sims, Spiteri et al. 2007, Smogorzewska, Matsuoka et al. 2007, Vaz, Hanenberg et al. 2010, Kim, Lach et al. 2011, Gille, Floor et al. 2012, Bogliolo, Schuster et al. 2013, Kupfer 2013). Additional FA genes may remain to be discovered. One FA gene, *FANCB*, demonstrates an X-linked recessive inheritance pattern, but the majority (fifteen of the sixteen known FA genes, representing ~98% of cases of FA) are inherited in an autosomal recessive fashion (Meetei, Levitus et al. 2004, Auerbach 2009, Oostra, Nieuwint et al. 2012). FA affects approximately one in 360,000 live births (Lin and Kutler 2013), and approximately one in 300 people are carriers of an FA mutation (D'Andrea 2010). Three of the FA genes account for ~85% of the disease. FANCA mutations are the most common and occur in ~60% of FA patients, while FANCC

mutations account for ~15% of cases and FANCG mutations account for ~10% of cases of FA (Taniguchi and D'Andrea 2006, Auerbach 2009). The other ~15% of FA patients have mutations in one of the other twelve genes, and the relative prevalence of mutations in each gene is 5% or less (Gille, Floor et al. 2012).

While FA is widely heterogenous in its presentation, the defining characteristics of FA include developmental abnormalities, pancytopenia progressing to bone marrow failure (BMF), and a high predisposition to a variety of cancers, particularly acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and squamous cell carcinomas (SCC) (Kutler, Singh et al. 2003, Bagby and Alter 2006, Masserot, Peffault de Latour et al. 2008, Auerbach 2009). Approximately 70% of FA patients have notable physical abnormalities which can aid in the diagnosis of FA (Giampietro, Verlander et al. 1997). Characteristic features include unique facial features, an absent thumb, and a deep cleft between the thumb and forefinger. Features which are less specific to FA include skeletal abnormalities, skin hyperpigmentation and café-au-lait spots, malformation of the reproductive organs and kidneys, small head, small eyes, and short stature (Bagby and Alter 2006, Alter 2008, D'Andrea 2010).

FA is typically diagnosed between five and ten years of age by the recognition of low blood cell counts in multiple lineages (Reuter, Medhurst et al. 2003, Oostra, Nieuwint et al. 2012). Pancytopenia is the first sign of FA in ~90% of cases. However, the onset of AML or another cancer can proceed pancytopenia (Velez-Ruelas, Martinez-Jaramillo et al. 2006, Alter, Rosenberg et al. 2007). Patients of the FANCD1/BRCA2 (breast cancer susceptibility 2) and

FANCN/PALB2 (partner and localizer of BRCA2) subtypes are considered to have a more severe form of FA and often develop AML, medulloblastoma, or Wilms' tumor during the first few years of life (Wagner, Tolar et al. 2004, Alter, Rosenberg et al. 2007, Reid, Schindler et al. 2007). FANCC patients with the Ashkenazi Jewish mutation (deletion of exon 4) also frequently develop AML at a young age (Auerbach 1997, Gillio, Verlander et al. 1997).

When FA was first discovered by the Swiss pediatrician Guido Fanconi in 1927, the underlying pancytopenia was untreatable, and FA was fatal as pancytopenia progressed to overt BMF (Fanconi 1927, Bagby and Alter 2006). The development of modern blood banking technology in the US in the early- to mid-twentieth century enabled the replacement of an FA patient's red blood cells and platelets, improving survival rates. However, FA patients were still highly susceptible to bacterial and fungal infections due to low white blood cell counts, and neutropenic infections became the major cause of death. With only the supportive therapy of blood product administration, few FA patients live into their twenties (Scagni, Saracco et al. 1998, D'Andrea, Dahl et al. 2002, Green and Kupfer 2009). Fortunately, treatment options for FA have improved over time.

Several treatment options currently exist for the hematopoietic defects in FA. Androgens and hematopoietic growth factors stimulate hematopoiesis and can be used to manage the symptoms of pancytopenia, but over time FA patients become refractory to these therapies (D'Andrea 2010). The most effective treatment for FA is hematopoietic stem cell transplantation (HSCT), which cures the hematopoietic manifestations of FA (MacMillan, Hughes et al. 2011).

The protocols for HSCT in FA were originally based on those for patients with acquired aplastic anemia. FA patients exhibited severe graft-versus-host disease (GVHD) and high toxicity, resulting in poor survival (Gluckman, Devergie et al. 1980, Gluckman, Devergie et al. 1983). It was discovered that FA patients are hypersensitive to irradiation and to cyclophosphamide, a DNA crosslinking agent (Berger, Bernheim et al. 1980a, Auerbach, Adler et al. 1983, Gluckman, Berger et al. 1984). The myeloablative regimen was modified by lowering the doses of irradiation and cyclophosphamide used with FA patients, resulting in improved survival (Gluckman, Berger et al. 1984). However, decreased myeloablation led to decreased engraftment. Fludarabine has been added to the standard regimen for myeloablative preparation for HSCT in FA patients. The result has been a dramatic improvement in patient outcomes, with the vast majority of patients exhibiting successful engraftment and low levels of toxicity from the myeloablative regimen (Aker, Varadi et al. 1999, Boulad, Gillio et al. 2000, Tan, Wagner et al. 2006, Yabe, Inoue et al. 2006, Chaudhury, Auerbach et al. 2008, Hamidieh, Alimoghaddam et al. 2011, Shimada, Takahashi et al. 2012). Furthermore, the use of T-cell depleted hematopoietic stem cells for transplantation has improved the rates of GVHD (Boulad, Gillio et al. 2000, Chaudhury, Auerbach et al. 2008, Huck, Hanenberg et al. 2008).

Using current regimens, HSCT is generally successful when an HLA-identical sibling donor is available (Balci, Akdemir et al. 2008, Huck, Hanenberg et al. 2008, Ayas, Saber et al. 2013). Transplants utilizing hematopoietic stem cells from bone marrow (BM), mobilized peripheral blood, and umbilical cord

blood from closely-matched related and unrelated donors have been performed successfully in FA patients (Aker, Varadi et al. 1999, Boulad, Gillio et al. 2000, MacMillan, Auerbach et al. 2000, de la Fuente, Reiss et al. 2003, Yabe, Inoue et al. 2006, Chaudhury, Auerbach et al. 2008). With current myeloablative preparatory regimens, transplantation with UCB or T-cell depleted BM both result in high levels of engraftment and relatively low levels of GVHD, when the HLA types are identical or only differ at a single locus (Tan, Wagner et al. 2006, Gluckman, Rocha et al. 2007).

Umbilical cord blood (UCB) transplantation and FA have a unique history. The first successful UCB transplants were performed in FA patients (Gluckman, Broxmeyer et al. 1989, Broxmeyer, Gluckman et al. 1990, Gluckman, Devergie et al. 1990). Furthermore, the first combined utilization of preimplantation genetic diagnosis and HLA matching was performed in the context of HSCT for FA (Verlinsky, Rechitsky et al. 2001). The best outcomes in FA patients result from HSCT with BM or UCB from an HLA-identical sibling donor (Huck, Hanenberg et al. 2008, MacMillan and Wagner 2010). However, most FA patients do not have an HLA-identical, non-FA sibling. Using in vitro fertilization with preimplantation genetic testing and HLA typing, parents of an FA patient can have an HLA-identical child without FA. UCB can be collected at delivery and transplanted into the sibling with FA. Two independent cases successfully utilizing this approach were reported (Bielorai, Hughes et al. 2004, Grewal, Kahn et al. 2004).

While HSCT cures the hematopoietic disease in FA patients, it does not decrease predisposition to solid tumors. Long-term follow-up studies indicate that

40% of transplanted FA patients will develop solid tumors within 15-20 years of transplant (Deeg, Socie et al. 1996). Prior to HSCT, AML is the most common cancer in FA patients. Afterward, SCC is the most common.

Because predisposition to cancer is a major feature of FA, preventive measures, cancer screening, and cancer treatment are important aspects of an FA patient's care (D'Andrea 2010). Cancer is difficult to treat in FA patients because they are hypersensitive to radiation and many chemotherapeutic agents including cyclophosphamide and cisplastin, the drugs commonly used to treat AML and SCC respectively (Green and Kupfer 2009, Scheckenbach, Wagenmann et al. 2012). A thorough understanding of the molecular pathogenesis of FA is needed and may enable the development of better treatment options for FA patients.

Known biological functions of the FA signaling network

The biomolecular study of FA is a relatively young field. The cloning of the first two FA genes, FANCC and FANCA, occurred in 1992 and 1996, respectively (Strathdee, Duncan et al. 1992, Strathdee, Gavish et al. 1992, Foe, Rooimans et al. 1996). FANCG became the third FA gene identified in 1998 (de Winter, Waisfisz et al. 1998). The discovery and cloning of novel FA genes has continued through the present time, and our molecular understanding of the pathogenesis of FA is continuing to evolve. Table 1-1 presents a complete list of the FA genes/proteins including years discovered and selected molecular features.

Gene	Other names	Year identi-fied	Chromo-somal location	Known functional motifs	Canonical FA group
FANCA		1996	16q24.3	NLS, NES	Core complex
FANCB		2004	Xp22.31	NLS	Core complex
FANCC		1992	9q22.3	None	Core complex
FANCE		2000	6p21.22	NLS	Core complex
FANCF		2000	11p15	none	Core complex
FANCG	*XRCC9*	1998	9p13	TPRs	Core complex
FANCL	*PHF9*	2003	2p16.1	E3 ligase	Core complex
FANCM		2005	14q21.3	ATPase, DNA translocase	Core complex
FANCD2		2001	3p25.3	none	ID complex
FANCI	*KIAA1794*	2007	15q25-26	none	ID complex
FANCD1	*BRCA2*	2002	13q12.13	BRC repeats	Downstream
FANCJ	*BACH1/BRIP1*	2005	17q22-24	ATPase, DNA helicase	Downstream
FANCN	*PALB2*	2007	16p12	WD40 domain	Downstream
FANCO	*RAD51C*	2010	17q23	RAD51 homolog	Downstream
FANCP	*SLX4*	2011	16p13.3	endonuclease	Downstream
FANCQ	*ERCC4/XPF4*	2013	16p13.12	endonuclease	Downstream

Table 1-1. Summary of known FA genes/proteins. This table summarizes basic information about the known FA genes and their protein products, including name, chromosomal location, and the year when each gene was identified as an FA gene. Additionally, known functional motifs of the FA proteins and their roles in the canonical DNA damage pathway are listed. This table was adapted from Table 1 in Yale J Biol Med. 86(4): 491–497 (Kupfer 2013). The year when each FA gene was identified was based on the earliest publication which could be found identifying the chromosomal location. In many cases, the FA gene's chromosomal location was unknown when the complementation group was named. In these cases, the publication year for positional cloning and identification of the chromosomal location is given. In cases where a previously known gene was subsequently identified as an FA protein, the year when the gene was published as a novel FA complementation group is used. The references used to determine the discovery year for each FA gene are given at the end of the first paragraph of the current chapter.

The FA proteins are known to cooperate with one another and with BRCA1 (breast cancer susceptibility protein 1) in a canonical pathway which participates in the repair of DNA interstrand crosslink (ICL) damage during the S-phase checkpoint (Grompe and D'Andrea 2001). The FA/BRCA DNA repair pathway is diagramed in Figure 1-1 on the next page and summarized in this and subsequent paragraphs. As the initial step of the canonical FA/BRCA pathway, eight of the FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) travel to the nucleus and assemble into a multiprotein core complex (CC) (Green and Kupfer 2009). The Fanconi anemia-associated proteins FAAP20, FAAP24, and FAAP100 are also part of the S-phase CC (Ciccia, Ling et al. 2007, Ling, Ishiai et al. 2007, Ali, Pradhan et al. 2012, Leung, Wang et al. 2012). FANCM has seven helicase domains which enable it to interact directly with chromatin (Meetei, Medhurst et al. 2005). It is believed that FANCM recognizes damaged DNA, recruits the other FA CC members to the nucleus, and acts as a scaffold for CC assembly (Kim, Kee et al. 2008, Deans and West 2011). FANCA, FANCB, and FANCE each have a nuclear localization sequence (NLS) (Lightfoot, Alon et al. 1999, de Winter, Leveille et al. 2000, Meetei, Levitus et al. 2004), and they are believed to help FANCG, FANCL, and FANCC respectively to translocate into the nucleus (Medhurst, Laghmani et al. 2006).

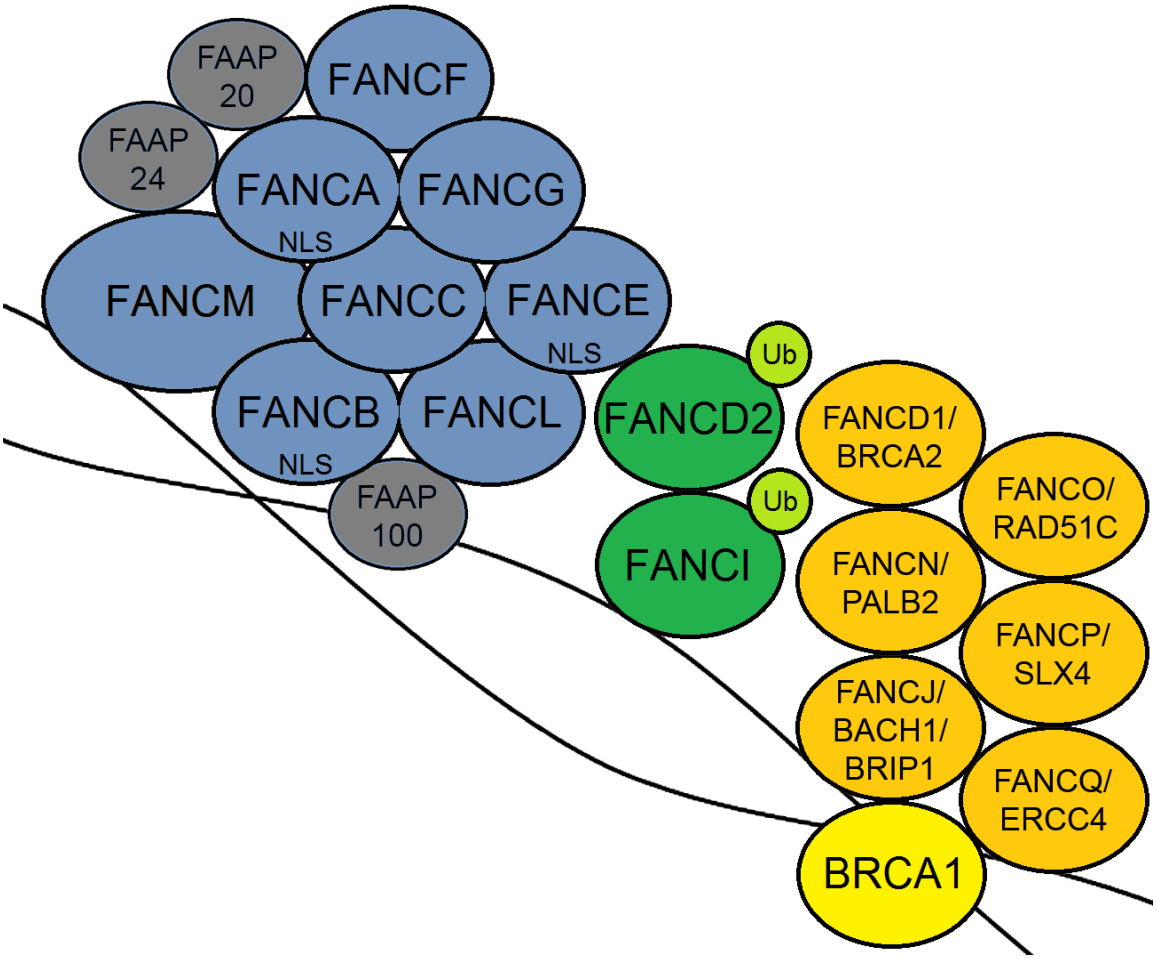


Figure 1-1. The canonical FA/BRCA DNA repair pathway responds to DNA interstrand crosslink (ICL) damage during interphase. FA proteins which comprise the core complex (CC) are shown in blue, and Fanconi anemia-associated proteins (FAAPs) which associate with the FA CC are depicted in gray. The FA CC, acting as a multisubunit ubiquitin ligase, activates FANCD2 and FANCI by adding a single ubiquitin moiety to each. The FANCD2/FANCI heterodimer (ID complex) is green, and ubiquitin groups (Ub) are depicted as light green circles attached to FANCD2 and FANCI. The six downstream FA proteins are gold, while BRCA1—which physically interacts with FANCA, with FANCJ/BACH1/BRIP1 and with FANCN/PALB2—is shown in yellow.

Once assembled, the nuclear FA CC acts as a multisubunit ubiquitin ligase for FANCD2 and FANCI (ID complex) (Alpi and Patel 2009). While FANCL has the catalytic E3 ubiquitin ligase activity (Meetei, de Winter et al. 2003) and FANCE mediates the interaction of the FA CC with FANCD2 (Pace, Johnson et al. 2002), each of the eight FA CC members is essential for the effective monoubiquitination of FANCD2 and FANCI (Green and Kupfer 2009). Monoubiquitination of FANCD2 on lysine 561 and FANCI on lysine 523 activates the ID complex, resulting in its translocation to damaged DNA. The downstream effectors of the ID complex are the remaining FA proteins (FANCD1/BRCA2, FANCJ/BACH1 [BRCA1-associated C-terminal helicase]/BRIP1 [BRCA1-interacting protein], FANCN/PALB2, FANCO/RAD51C [Rad51 homolog C], FANCP/SLX4 [structure-specific endonuclease subunit SLX4], and FANCQ/ERCC4 [excision repair cross-complementing 4] (Kottemann and Smogorzewska 2013).

When FANCD2 and FANCI translocate to damaged chromatin, they form nuclear foci along with their downstream effectors (Kim and D'Andrea 2012). The downstream FA protein FANCJ, like FANCM, is a DNA helicase with the ability to directly interact with DNA (Wu, Sommers et al. 2012). FANCJ binds to the carboxy-terminal domain of BRCA1 (breast cancer susceptibility 1) and promotes BRCA1's DNA repair activity (Cantor, Bell et al. 2001). The FA proteins interact with numerous other proteins known to play roles in sensing and responding to DNA damage, including BRCA1, ATM (Ataxia Telangiectasia-mutated), ATR (ATM-Rad3-related), CHK1 (checkpoint kinase 1), CHK2 (checkpoint kinase 2),

NBS1 (Nijmegen breakage syndrome 1), RAD50, and BLM (Bloom syndrome) (Chen, Silver et al. 1998, Garcia-Higuera, Taniguchi et al. 2001, Nakanishi, Taniguchi et al. 2002, Rosselli, Briot et al. 2003, Sridharan, Brown et al. 2003, Pichierri, Franchitto et al. 2004, Wang and D'Andrea 2004, Wang, Kennedy et al. 2007, Kupfer 2013).

The current model for ICL repair integrates roles for the FA proteins in nucleotide excision, translesion DNA synthesis, and homologous recombination. In this model, first, endonucleases unhook the ICL. Then, error-prone translesion synthesis polymerases replicate over the unhooked ICL. Finally, homologous recombination is performed (Kim and D'Andrea 2012). FANCP acts as a scaffold protein for a number of endonucleases which may function in the removal of DNA crosslinks (Kim, Spitz et al. 2013). The FA core complex recruits the translesion synthesis polymerase REV1 to nuclear foci existing at sites of DNA ICL damage through the interaction of FAAP20 with REV1 (Kim, Yang et al. 2012). Finally, the downstream effectors of the FA/BRCA pathway—BRCA1, FANCD1/BRCA2, FANCJ/BACH1/BRIP1, FANCN/PALB2, and FANCO/RAD51C—are all known to play important roles in the repair of DNA double strand breaks via homologous recombination (Moynahan, Pierce et al. 2001, French, Masson et al. 2002, Litman, Peng et al. 2005, Sy, Huen et al. 2009, Zhang, Ma et al. 2009). When performed in the context of DNA repair, homologous recombination generally produces non-crossover products which are identical to the original DNA sequences (Daley, Kwon et al. 2013). Thus, homologous recombination

represents a process which the FA pathway could use to restore damaged chromatin to its pre-damaged state (Kim and D'Andrea 2012).

Because the FA proteins play a key role in the repair of DNA crosslink damage, FA-deficient cells are hypersensitive to DNA crosslinking agents (Centurion, Kuo et al. 2000, Su and Huang 2011). This hypersensitivity is the basis for the diagnosis of FA. In the chromosome breakage test, which is the gold standard diagnostic test for FA, stimulated peripheral T lymphocytes from a patient suspected to have FA are challenged with mitomycin C (MMC) or diepoxybutane (Oostra, Nieuwint et al. 2012). Then the cells are plated on glass slides as metaphase spreads and the number of chromosome breaks are quantified. A positive chromosome breakage test results in a diagnosis of FA (Auerbach 2009). In some cases of FA, somatic reversion (spontaneous gene-correction) occurs in the patient's hematopoietic cells and the chromosome breakage test is negative (Hirschhorn 2003). In these cases, a skin biopsy should be performed and the test repeated on cultured skin fibroblasts (Oostra, Nieuwint et al. 2012). Following a diagnosis of FA, the subtype can be determined by complementation analysis and mutation screening (Tamary and Alter 2007, de Winter and Joenje 2009, Ameziane, Sie et al. 2012).

A role for the FA proteins in the S-phase repair of DNA ICL damage is well established, and many follow-up studies have provided insight into the specific mechanistic roles of the individual FA proteins in this process (Bridge, Vandenberg et al. 2005, de Winter and Joenje 2009, Sato, Ishiai et al. 2012, Shukla, Solanki et al. 2013). However, the FA proteins are believed to have

additional functions beyond their canonical roles in S-phase DNA damage repair (Kee and D'Andrea 2010). Physical interaction screens and functional studies have linked the FA proteins to many other cellular processes, including transcription, cell signaling, cellular transport, apoptosis, cytokine signaling, oxidative metabolism, aldehyde metabolism, and centrosome biology (Reuter, Medhurst et al. 2003, Langevin, Crossan et al. 2011, Meier and Schindler 2011, Garaycoechea, Crossan et al. 2012, Kaddar and Carreau 2012, Kim, Hwang et al. 2013, Zou, Tian et al. 2013). Ongoing studies continue to elucidate these additional roles of the FA pathway (unpublished data, 25th Annual Fanconi Anemia Research Fund Scientific Symposium 2013).

The FA signaling network, aneuploidy, and cancer

The sixteen members of the FA signaling pathway act as tumor suppressors by functioning in the maintenance of genomic integrity (Mosedale, Niedzwiedz et al. 2005, Schlacher, Wu et al. 2012, Pickering, Zhang et al. 2013). Genomic instability in FA-deficient cells is characterized by chromosome breaks and gross aneuploidy (Berger, Bernheim et al. 1980b, Berger, Le Coniat et al. 1993, Tutt, Gabriel et al. 1999, D'Andrea 2003, van der Heijden, Yeo et al. 2003, Mehta, Harris et al. 2010). FA patients have a high predisposition to cancer, especially acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and squamous cell carcinomas (SCC) (Alter, Giri et al. 2010). Moreover, somatic alterations in the FA genes have been implicated in a significant proportion of cancers arising in the general population, particularly pancreatic, breast, and

ovarian cancers (Schutte, da Costa et al. 1995, van der Heijden, Yeo et al. 2003, Jones, Hruban et al. 2009). While the canonical role of the FA pathway in DNA interstrand crosslink damage is believed to contribute, the aneuploidy and oncogenesis resulting from inactivation of the FA pathway are incompletely understood (Kee and D'Andrea 2010).

Aneuploidy and FA

Clinical studies dating back to the 1970s have examined the cytogenetics profiles of bone marrow (BM) aspirates from pre-leukemic and leukemic FA patients (Rochowski, Olson et al. 2012). These studies have reported the observation of complex gross chromosomal aberrations in FA-deficient BM cells and have associated the presence of aneuploidy with progression to MDS and AML (Berger, Le Coniat et al. 1993, Alter, Caruso et al. 2000, Cioc, Wagner et al. 2010, Mehta, Harris et al. 2010). In the non-FA population, AML commonly carries specific balanced translocations such as t(8; 21) and translocations involving inv(16). It is notable that these and other AML-specific nonrandom balanced chromosomal rearrangements have never been reported in FA patients (Auerbach and Allen 1991, Rochowski, Olson et al. 2012). Rather, the types of gross aneuploidy observed in BM cells taken from FA patients are often random and unbalanced. Gains and losses of partial and complete chromosomes are frequently seen in FA, as well as translocations, which can be either balanced or unbalanced (Mehta, Harris et al. 2010, Meyer, Neitzel et al. 2012). Certain gross chromosomal aberrations occur more frequently than others and have been

correlated with clonal evolution in FA (Tonnies, Huber et al. 2003, Cioc, Wagner et al. 2010, Mehta, Harris et al. 2010). Additionally, the aneuploidy in FA patients is frequently complex, with multiple aberrations existing in a single clone (Auerbach and Allen 1991). Interestingly, multiple clones carrying different forms of aneuploidy can exist simultaneously, with some clones only transiently detectable (Alter, Scalise et al. 1993, Alter, Caruso et al. 2000). Gains and losses, which are inherently unbalanced types of chromosomal rearrangements, are believed to occur throughout the process of clonal evolution in the FA patient (Alter, Caruso et al. 2000, Meyer, Neitzel et al. 2012).

The most commonly reported chromosomal aberrations in FA are gains involving chromosome 1q and chromosome 3q, and deletions involving chromosome 7 (Berger and Jonveaux 1996, Cioc, Wagner et al. 2010). Gains of 1q are believed to occur in the early stages of clonal evolution as they are often found in the absence of other chromosomal rearrangements in FA BM which remains morphologically normal (Meyer, Neitzel et al. 2012). Gains of 3q have been associated with the development of MDS and AML in the FA patient (Tonnies, Huber et al. 2003, Mehta, Harris et al. 2010, Quentin, Cuccuini et al. 2011). Although balanced chromosomal rearrangements involving 3q are often found in AML in the non-FA population, gains of 3q are much more frequent in FA (Lugthart, van Drunen et al. 2008, Lugthart, Groschel et al. 2010, Rochowski, Olson et al. 2012). Monosomy 7 has also been associated with the development of MDS and AML in the FA patient and frequently occurs in more advanced clones carrying a complex karyotype (Kardos, Baumann et al. 2003, Mehta,

Harris et al. 2010). However, all three of the examples given—gains of 1q, gains of 3q, and deletions of part or all of chromosome 7—can be found accompanied by other forms of aneuploidy (Meyer, Neitzel et al. 2012). The presence of clones containing complex random and nonrandom aneuploidy in the BM of FA patients has been correlated with progression to MDS and AML (Alter, Caruso et al. 2000, Cioc, Wagner et al. 2010).

Cancer predisposition in FA patients

At least 20% of FA patients will develop cancer at some point during their lifetimes (Alter 2003, Kutler, Singh et al. 2003). The FA patient is predisposed to multiple types of hematopoietic malignancies and solid tumors, most commonly acute myeloid leukemia (AML) and squamous cell carcinoma (SCC) (Rosenberg, Alter et al. 2008). A patient with FA who survives to the age of 40 has an actuarial risk of 33% for developing a hematopoietic malignancy and an actuarial risk of 28% for developing a solid tumor of some kind (Kutler, Singh et al. 2003). While the most common hematopoietic malignancies in FA patients are myeloid (AML and MDS), FA patients can also develop acute lymphocytic leukemia (ALL) (Mushtaq, Wali et al. 2012, Shah, John et al. 2013). Solid tumors which have been found in FA patients include head and neck SCC, gynecologic SCC, esophageal carcinoma, liver tumors, brain tumors, kidney tumors, and breast cancer (Alter 2003, Rosenberg, Alter et al. 2008). Embryonal tumors such as medulloblastoma (a type of brain cancer) and Wilms' tumor (a type of kidney cancer) are especially common in FA patients with mutations in the

FANCD1/BRCA2 and FANCN/PALB2 genes (Alter, Rosenberg et al. 2007, Reid, Schindler et al. 2007). Patients of these two FA subtypes often develop embryonal tumors and AML within the first few years of life (Wagner, Tolar et al. 2004, Alter, Rosenberg et al. 2007, Reid, Schindler et al. 2007).

AML is the single most common type of cancer in FA patients (Auerbach 1992, Alter 1996), and it is usually of the M1, M2, M3, or M4 subtype (acute myeloblastic leukemia with maturation, acute myeloblastic leukemia without maturation, acute promyelocytic leukemia, or acute myelomonocytic leukemia respectively) (Velez-Ruelas, Martinez-Jaramillo et al. 2006). Random aneuploidy is almost universally present, including large numbers of translocations, insertions, and deletions (Auerbach and Allen 1991, Alter 1992). AML in FA patients is difficult to treat. Alkylating agents such as cytoxan are standard therapy for AML, but FA patients are hypersensitive to alkylating agents and exhibit marked toxicity. Furthermore, even though lower doses of chemotherapeutic agents are used, FA patients are highly susceptible to secondary malignancies (Green and Kupfer 2009).

FA patients develop head and neck SCC and gynecologic SCC at an increasing rate as they grow older (Alter 2003, Alter, Greene et al. 2003, Rosenberg, Greene et al. 2003, Rosenberg, Alter et al. 2008). Additionally, FA patients may have an exacerbated risk for developing SCC after receiving irradiation and chemotherapeutic agents to prepare for a transplant or to treat a primary malignancy (Millen, Rainey et al. 1997, Rosenberg, Alter et al. 2005, Rosenberg, Socie et al. 2005, Masserot, Peffault de Latour et al. 2008). Random

aneuploidy is a major feature of head and neck SCC in FA patients and in the general population (Bockmuhl and Petersen 2002, van Zeeburg, Snijders et al. 2005). FA patients are hypersensitive to cisplatin and radiation, which are commonly used to treat SCC in the general population. Even with lower doses and local administration, cisplatin and radiation result in high levels of toxicity in FA patients. Thus, early detection and early surgical removal are the most important aspects of treatment for head and neck SCC in patients with FA (Scheckenbach, Wagenmann et al. 2012).

FA genes in sporadic and inherited cancer

In addition to their role in cancer predisposition in the FA patient, a number of FA genes are cancer susceptibility genes which have been identified in families with a history of inherited cancer. A large subset of the FA genes double as breast, ovarian, and/or pancreatic cancer susceptibility genes (Lancaster, Wooster et al. 1996, White, Held et al. 2001, Hahn, Greenhalf et al. 2003, Rogers, van der Heijden et al. 2004, Couch, Johnson et al. 2005, Seal, Thompson et al. 2006, Rahman, Seal et al. 2007, van der Groep, Hoelzel et al. 2008, Jones, Hruban et al. 2009, Meindl, Hellebrand et al. 2010, Pelttari, Heikkinen et al. 2011, Rafnar, Gudbjartsson et al. 2011). Furthermore, epigenetic inactivation of FA genes has been detected in a number of malignancies. Table 1-2 summarizes the role of FA genes in cancer predisposition in the non-FA, general population.

Gene	Other names	Cancer susceptibility in the non-FA, general population (heterozygous germline mutations, acquired mutations, or acquired epigenetic inactivation)	Canonical FA group
FANCA		AML	Core complex
FANCB			Core complex
FANCC		Pancreatic cancer	Core complex
FANCE			Core complex
FANCF		Ovarian cancer (>30%), breast cancer, AML, SCC, multiple other cancers	Core complex
FANCG	*XRCC9*	Pancreatic cancer	Core complex
FANCL	*PHF9*		Core complex
FANCM			Core complex
FANCD2		Breast cancer	ID complex
FANCI	*KIAA1794*		ID complex
FANCD1	*BRCA2*	Breast, ovarian, and pancreatic cancer	Downstream
FANCJ	*BACH1/ BRIP1*	Breast and ovarian cancer	Downstream
FANCN	*PALB2*	Breast, ovarian, pancreatic, and prostate cancer	Downstream
FANCO	*RAD51C*	Breast and ovarian cancer	Downstream
FANCP	*SLX4*		Downstream
FANCQ	*ERCC4/ XPF4*		Downstream

Table 1-2. Involvement of FA genes in cancer predisposition in the non-FA, general population.

A large subset of the FA proteins which function as downstream effectors in the FA DNA damage repair pathway—FANCD1/BRCA2, FANCJ/BACH1/BRIP1, FANCN/PALB2, and FANCO/RAD51C—are known susceptibility genes for breast and ovarian cancer (Lancaster, Wooster et al. 1996, Wagner, Tolar et al. 2004, Seal, Thompson et al. 2006, Rahman, Seal et al. 2007, Tischkowitz, Xia et al. 2007, Akbari, Tonin et al. 2010, D'Andrea 2010, Levy-Lahad 2010, Meindl, Hellebrand et al. 2010, Zheng, Zhang et al. 2010, Clague, Wilhoite et al. 2011, Pelttari, Heikkinen et al. 2011, Rafnar, Gudbjartsson et al. 2011, Vuorela, Pylkas et al. 2011, Thompson, Boyle et al. 2012). Mutations in FANCD1/BRCA2 and FANCN/PALB2 are also responsible for a large percentage of inherited pancreatic cancer (White, Held et al. 2001, Hahn, Greenhalf et al. 2003, van der Heijden, Yeo et al. 2003, Jones, Hruban et al. 2009), and FANCN/PALB2 has additionally been implicated in familial prostate cancer (Erkko, Xia et al. 2007, Tischkowitz, Sabbaghian et al. 2008, Pakkanen, Wahlfors et al. 2009).

Several upstream FA proteins may also play roles in cancer predisposition in the non-FA, general population. Germline and sporadic mutations in FANCC and FANCG have been found to play a role in young-onset pancreatic cancer (van der Heijden, Yeo et al. 2003, Rogers, van der Heijden et al. 2004, Couch, Johnson et al. 2005). Furthermore, decreased expression or complete epigenetic silencing of FA genes has been observed in several types of cancer. FANCA may be deleted or expressed at a lower level in AML (Xie, de Winter et al. 2000, Tischkowitz, Morgan et al. 2004). FANCD2 has been found to be silenced in

some sporadic and hereditary breast cancers (van der Groep, Hoelzel et al. 2008). Finally, FANCF is frequently silenced in multiple types of cancer (Taniguchi 2009).

Epigenetic silencing of FANCF, resulting in failure to monoubiquitinate FANCD2, have been observed in a wide variety of cancers occurring in the general population. Epigenetic silencing of FANCF occurs by hypermethylation. When CpG islands in FANCF's promoter region are hypermethylated, the FANCF gene is no longer transcribed. Because the FA core complex member FANCF is absent, monoubiquitination of FANCD2 fails to occur (Kalb, Neveling et al. 2006). Silencing of FANCF by hypermethylation has been observed in up to one third of ovarian cancers (Olopade and Wei 2003, Taniguchi, Tischkowitz et al. 2003, Wang, Li et al. 2006, Lim, Smith et al. 2008). FANCF silencing has also been observed in acute myeloid leukemia, breast cancer, cervical cancer, non-small cell lung cancer, head and neck SCC, granulosa cell tumors of the ovary, testicular germ cell tumors (non-seminoma), and bladder cancer (Xie, de Winter et al. 2000, Tischkowitz, Ameziane et al. 2003, Dhillon, Shahid et al. 2004, Koul, McKiernan et al. 2004, Marsit, Liu et al. 2004, Narayan, Arias-Pulido et al. 2004, Neveling, Kalb et al. 2007, Wei, Xu et al. 2008).

Cancer predisposition in FA knockout mice

A number of single knockout FA murine models have been developed and used to study cancer predisposition in FA. While some single knockout FA mice (Fanca-/-, Fancc-/-, Fancd2-/-, and Fancd1/Brca2 conditional knockout mice)

develop tumors, they do not spontaneously develop the types of cancer observed in FA patients (Moynahan 2002, Taniguchi and D'Andrea 2006). The Fancd2-/- mouse develops a broad spectrum of epithelial tumors, including ovarian, gastric, and hepatic adenoma; ovarian, mammary, and lung adenocarcinoma; bronchoalveloar carcinoma; and hepatocellular carcinoma. One Fancd2-/- mouse with epithelial cancer also developed B-cell lymphoma (Houghtaling, Timmers et al. 2003). Similarly, lymphoma, sarcoma, and ovarian granulosa cell tumors were reported in the Fanca-/- mouse (Wong, Alon et al. 2003), and mammary adenocarcinoma and histiocytic sarcoma were reported in elderly Fancc-/- mice (Carreau 2004).

Since Fancd1/Brca2-/- mice die in utero, a number of murine models have been generated utilizing partial deletion or conditional knockout strategies (Moynahan 2002). A Brca2 mutant mouse model homozygous for deletion of exon 27 develops carcinomas, adenomas, sarcomas, and lymphomas in a variety of locations including the stomach, lung, breast, and ovary. Exon 27 is essential for Brca2's interaction with Rad50, a protein involved in the repair of DNA double-strand breaks (McAllister, Bennett et al. 2002). Conditional knockout of Fancd1/Brca2 in mammary epithelium also results in tumorigenesis. Breast cancer developed in 77% of Brca2 conditional knockout mice compared with 0% of wild-type (WT) mice. Furthermore, multiple tumors were present in 50% of the mice which developed breast cancer (Ludwig, Fisher et al. 2001).

While existing single knockout FA mice do not spontaneously develop hematopoietic malignancies and BMF, one or more types of double knockout

mice may. The *Fancc-/-; Fancg-/-* mouse which our laboratory previously developed was the first murine model of FA to spontaneously develop the MDS, AML, and BMF which are major features of the human disease. This study assessed the predisposition to cancer in the absence of murine Fancc and Fancg and examined the morphologic architecture, cytogenetic status, and transcriptomal profile of FA-deficient BM (Pulliam-Leath, Ciccone et al. 2010). The findings of hypoplastic bone marrow, aneuploidy, and SAC dysregulation in the *Fancc-/-; Fancg-/-* murine model of FA are summarized in Figure 1-2.

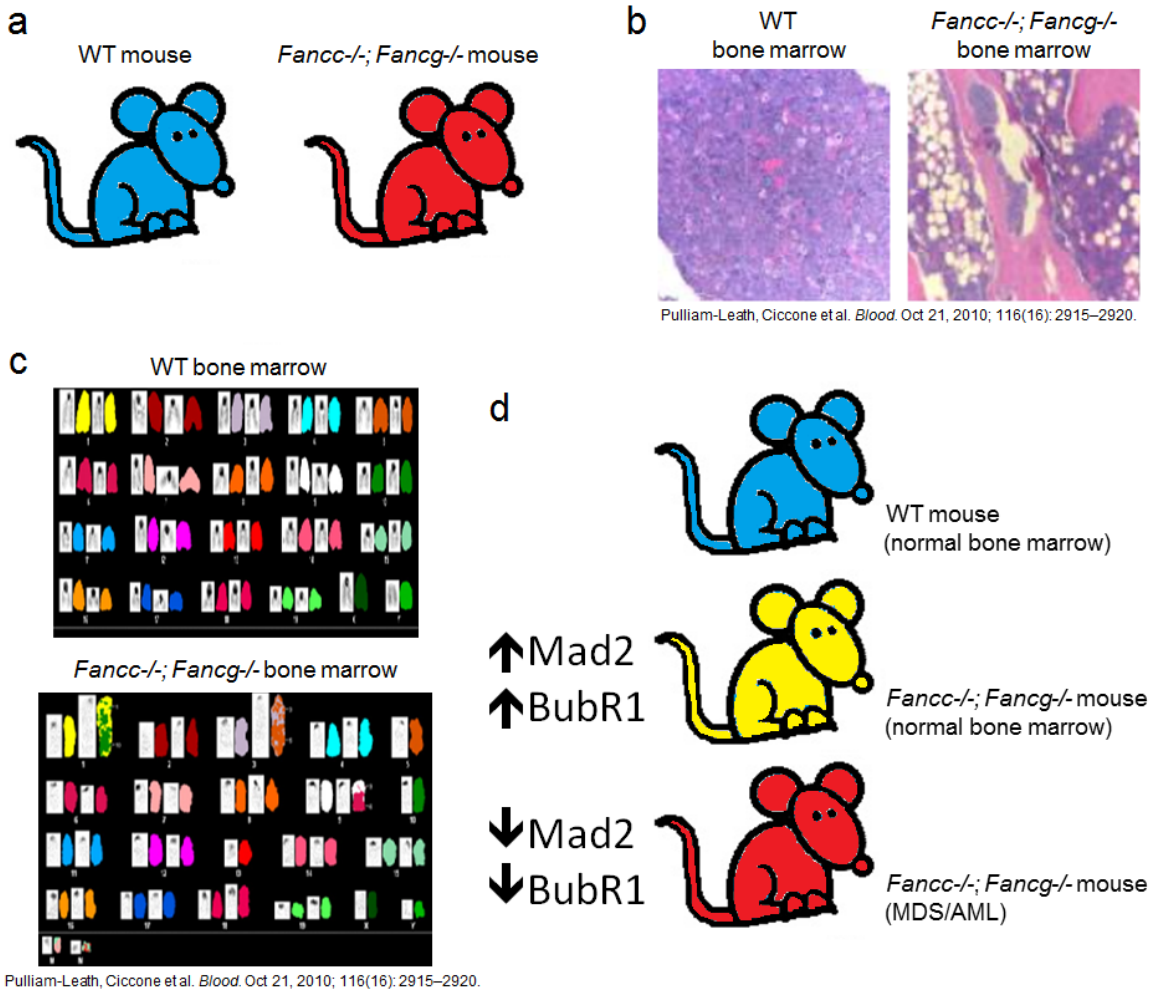


Figure 1-2. Hypocellularity, aneuploidy, and SAC dysregulation are observed in the bone marrow of *Fancc-/-; Fancg-/-* mice. a) WT and *Fancc-/-; Fancg-/-* mice. **b)** Hypocellular bone marrow (BM) is observed in *Fancc-/-; Fancg-/-* mice, while wild-type (WT) mice have BM with normal cellularity. **c)** BM cells from *Fancc-/-; Fancg-/-* mice exhibit gross aneuploidy upon spectral karyotyping, while WT mice have a normal karyotype. **d)** When the transcriptomal profile of BM cells from WT and *Fancc-/-; Fancg-/-* mice was analyzed utilizing a gene-chip based assay, dysregulation of known regulators of the mitotic SAC was observed in BM from *Fancc-/-; Fancg-/-* mice compared with WT mice. Bone marrow from *Fancc-/-; Fancg-/-* mice with normal bone marrow architecture exhibited increased expression of the SAC regulators Mad2 and BubR1 compared with WT mice, and bone marrow from *Fancc-/-; Fancg-/-* mice with myelodysplastic bone marrow exhibited decreased expression of the SAC regulators Mad2 and BubR1 compared with WT mice.

Clinical studies examining the BM from pre-leukemic and leukemic FA patients have noted that gross aneuploidy and myelodysplasia precede the development of leukemia in the FA patient (Berger, Bernheim et al. 1980b, Tonnies, Huber et al. 2003, Mehta, Harris et al. 2010, Quentin, Cuccuini et al. 2011, Meyer, Neitzel et al. 2012). In previous work utilizing the *Fancc-/-; Fancg-/-* murine model, my laboratory confirmed that there is a correlation between gross aneuploidy, myelodysplasia, and leukemia in FA (Pulliam-Leath, Ciccone et al. 2010). Additionally, dysregulation of the known mitotic spindle assembly checkpoint (SAC) regulators Mad2 and BubR1 was observed in a gene chip-based genome-wide transcriptomal assay performed in BM cells taken from this mouse model (A. Pulliam-Leath, S. Ciccone, G. Nalepa, G. Bagby, D. W. Clapp, unpublished data). Based on this result, we hypothesized that aneuploidy and oncogenesis may result from defective regulation of mitosis in FA-deficient cells. In particular, we hypothesized that the human FA proteins are essential for the proper functioning of the mitotic SAC and that this may be a major mechanism by which the FA pathway protects the integrity of the genome.

Maintaining the genome

Proper execution of the cell cycle is essential for cells to maintain the integrity of the genome (Shackelford, Kaufmann et al. 1999). Cell cycle checkpoints exist throughout interphase so that the cell can monitor DNA and repair any damage prior to the onset of mitosis (Branzei and Foiani 2008). During mitosis, the replicated DNA divides equally into daughter cells through a process called chromosome segregation. Chromosome segregation is regulated in order to ensure stable transmission of genetic material to daughter cells. The M-phase checkpoint, or spindle assembly checkpoint (SAC), is the key mitotic cell cycle checkpoint which protects cells from the development of aneuploidy by regulating chromosome segregation. Importantly, genomic instability due to weakened activity of the mitotic SAC may contribute to the process of malignant transformation (Kops, Weaver et al. 2005). The subsections below summarize the basic mechanics of the cell cycle, explain in detail the mechanisms of the mitotic SAC, and articulate the importance of the mitotic SAC in the maintenance of genomic integrity.

The cell cycle

Multicellular organisms complete the cell cycle to grow and replace older or damaged cells. The cell cycle has multiple discrete phases—gap/growth 1 (G1), synthesis (S), gap/growth 2 (G2), mitosis (M), and cytokinesis. The G1-, S-, and G2-phases are collectively referred to as interphase, while mitosis and cytokinesis are collectively referred to as cell division. In G1, cell growth occurs

through expansion of the cell's cytoplasm and organelle pool. G1 is the longest phase and often varies in length. From G1, the cell can enter a quiescent, non-cycling state called G0. The cell replicates its DNA and centrosomes during S and prepares for mitosis in G2. Then, the cell divides. Nuclear division occurs during mitosis, and cytoplasmic division occurs during cytokinesis. When a single cell has completed a full round of the cell cycle, two genetically identical daughter cells are the result. By definition, mitosis is the process by which a single parent cell divides its replicated genome equally in order to produce two genetically identical daughter cells (Nicolini 1975, Oshima and Campisi 1991, Norbury and Nurse 1992, O'Connor 2010). A schematic diagram of the cell cycle, mitosis, and cytokinesis appears in Figure 1-3. The subsequent paragraphs summarize the phases of mitosis and cytokinesis and discuss the role of cell cycle checkpoints in the maintenance of genomic integrity.

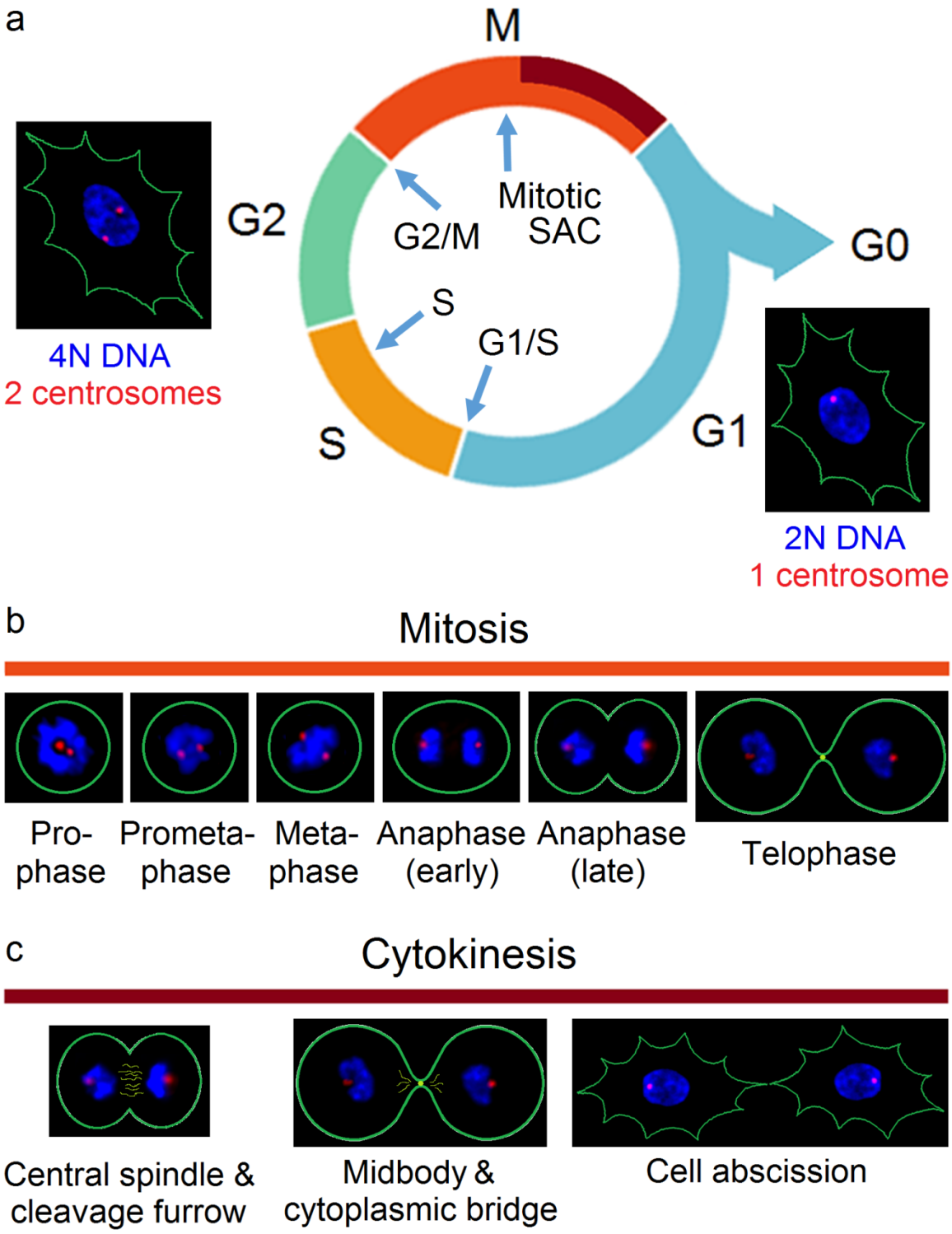


Figure 1-3. The cell cycle, mitosis, and cytokinesis. a) The phases of the cell cycle are G1/G0, S, G2, and M. Cell cycle phases are labeled on the outside of the circle, and cell cycle checkpoints are labelled on the inside. In the examples of G1 and G2 cells which are shown, DNA is blue, centrosomes are red, and cell borders are outlined in green. **b)** Phases of mitosis. **c)** Stages of cytokinesis.

Mitosis has five phases: prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, the dividing cell's nuclear envelope begins to break down and its chromatin begins to condense. Kinetochores form at the centromere region of each sister chromatid, centrosomes migrate to opposing poles of the cell, and spindle nucleation begins. Prometaphase onset is marked by the sudden dissolution of the nuclear envelope, which allows the spindle to access the sister chromatid pairs. Dynamic lengthening and shortening of spindle microtubules enables spindle microtubules to find and attach to kinetochores located at the centromere region of each sister chromatid. As sister chromatids begin attaching to the spindle via their kinetochores and as dynamic lengthening and shortening of spindle microtubules continues, sister chromatid pairs migrate to the center of the dividing cell.

By the start of metaphase, bi-oriented kinetochore-spindle attachments have formed on each sister chromatid pair. The metaphase plate exists when sister chromatid pairs have trafficked to the center of the cell and are neatly aligned halfway between the opposing spindle poles. Next, anaphase is initiated. Sister chromatids become separated from their partners and are pulled toward opposing spindle poles as the mitotic spindle shortens. Telophase starts when the dividing chromosomes reach the opposing spindle poles. Now, chromatin de-condenses, nuclear envelopes re-form, and a single dividing cell becomes two interphase cells (Thyberg and Moskalewski 1998, Mitchison and Salmon 2001, O'Connor 2008). The phases of mitosis are diagramed in Figure 1-3b.

Cytokinesis, like mitosis, has multiple stages. The stages of cytokinesis include central spindle formation, cleavage furrow initiation and ingression, midbody and cytoplasmic bridge formation, and cell abscission (D'Avino, Savoian et al. 2005, Montagnac, Echard et al. 2008, Fededa and Gerlich 2012). Cytokinesis is temporally coordinated with mitosis. Cleavage furrow initiation follows the initiation of chromosome segregation, and cell abscission is coordinated with mitotic exit (Seshan and Amon 2004, Kops, Weaver et al. 2005, Pines 2006, Sullivan and Morgan 2007, Fededa and Gerlich 2012). The stages of cytokinesis are diagramed in Figure 1-3c.

Maintenance of the genome is a crucial aspect of the cell cycle which is ensured by the existence of cell cycle checkpoints. Interphase DNA damage checkpoints (G1/S checkpoint, S-phase checkpoint, and G2/M checkpoint) sense DNA damage and halt cell cycle progression until the DNA damage is repaired. In this way, the genome is protected during interphase. Mitosis is the part of the cell cycle concerned with the accurate segregation of genetic material, and the mitotic checkpoint plays a crucial role in preserving genomic integrity by ensuring that each daughter cell receives a full, identical complement of DNA at the conclusion of the cell cycle (Elledge 1996, Shackelford, Kaufmann et al. 1999). The major cell cycle checkpoints are indicated in the diagram of the cell cycle in Figure 1-3a.

The mitotic spindle assembly checkpoint

The mitotic checkpoint, or spindle assembly checkpoint (SAC), monitors kinetochore-spindle attachment in order to guarantee accurate chromosome segregation (Meraldi, Draviam et al. 2004). The activating signal for the SAC is the presence of unattached kinetochores during the early phases of mitosis (Rieder, Schultz et al. 1994, Rieder, Cole et al. 1995). During prophase and prometaphase, several key SAC effectors are recruited to unattached kinetochores and form the mitotic checkpoint complex (MCC). The presence of the MCC at one or more unattached kinetochores delays the onset of anaphase by preventing activation of the anaphase promoting complex/cyclosome (APC/C) (Chen, Waters et al. 1996, Taylor, Ha et al. 1998, Waters, Chen et al. 1998, Sudakin, Chan et al. 2001).

The kinetochore is a transient subcellular structure which plays a key role in mitosis by mediating the SAC. The kinetochore has two parts, the inner kinetochore and the outer kinetochore, and is comprised of at least 80 individual proteins. The inner kinetochore is the stable, structural part of the kinetochore, and the outer kinetochore is the site of microtubule binding and SAC activity (Cheeseman and Desai 2008, Santaguida and Musacchio 2009). When a kinetochore is unattached to the mitotic spindle, the MCC is present at the kinetochore and the SAC is active. A schematic representation of an early mitotic cell containing an unattached kinetochore appears in Figure 1-4a, and the structure of an unattached kinetochore is depicted in Figure 1-4b. The structural organization of the kinetochore is described in more detail in the next paragraph.

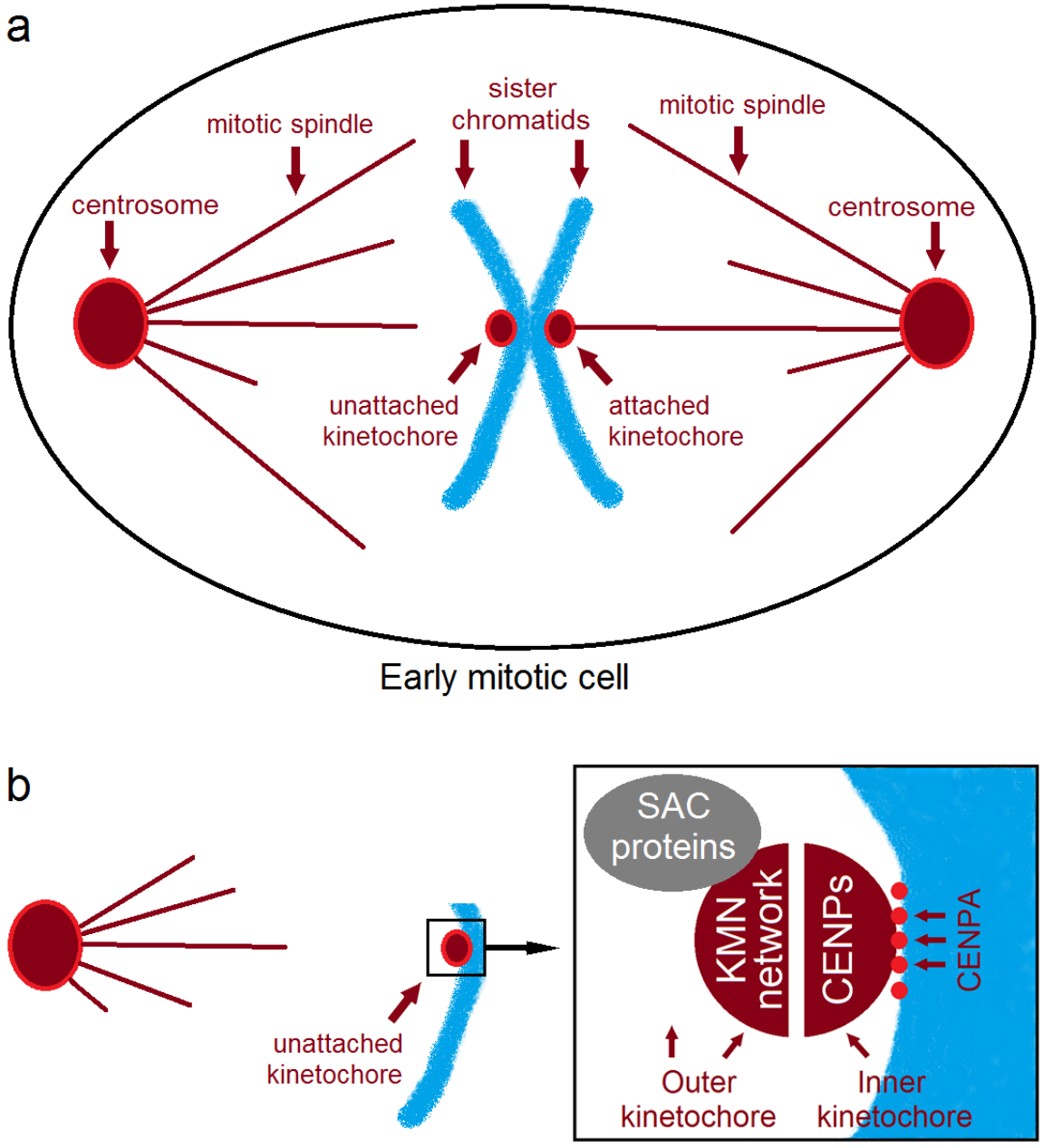


Figure 1-4. The presence of an unattached kinetochore in an early mitotic cell results in recruitment of SAC proteins and activation of the mitotic SAC. a) Schematic of an early mitotic cell with an unattached kinetochore. The centrosomes, mitotic spindle, sister chromatids, and kinetochores are labeled. **b)** Schematic depicting the structural organization of an unattached kinetochore. SAC proteins including the members of the mitotic checkpoint complex (MCC) are recruited to the outer kinetochore during the early phases of mitosis.

At the onset of mitosis, the kinetochore forms at the centromere region of each sister chromatid. As the first step, centromere protein A (CENPA), a modified histone H3 variant, is recruited to nucleosomes in centromeric chromatin (Palmer, O'Day et al. 1991, Sullivan, Hechenberger et al. 1994). CENPA recruits the sixteen members of the constitutive centromeric-associated network (CCAN) (CENPC, -H, -I, -K, -L, M, -N, -O, -P, -Q, -R, -S, -T, -U, -W, and -X) to form the inner kinetochore. Next, the CCAN recruits the KMN network—including the KNL1 protein and the Mis12 and Ndc80 protein complexes—to the outer kinetochore (Perpelescu and Fukagawa 2011). Through its interaction with CENPC, the Mis 12 complex mediates binding of the KMN network to the CCAN (Screpanti, De Antoni et al. 2011). KNL1 and the Ndc80 complex interact with spindle microtubules (Cheeseman, Chappie et al. 2006, Wan, O'Quinn et al. 2009, Alushin, Musinipally et al. 2012). Upon binding to the spindle, KNL1 plays a role in SAC silencing at the kinetochore (Espeut, Cheerambathur et al. 2012). Prior to that, KNL1 facilitates the SAC by recruiting SAC proteins, including members of the mitotic checkpoint complex (MCC), to the outer kinetochore (Krenn, Wehenkel et al. 2012, Shepperd, Meadows et al. 2012, Yamagishi, Yang et al. 2012, Varma, Wan et al. 2013, Krenn, Overlack et al. 2014).

SAC proteins are sequentially recruited to the outer kinetochore during mitosis (Jablonski, Chan et al. 1998, Taylor, Ha et al. 1998, Sharp-Baker and Chen 2001, Johnson, Scott et al. 2004). BUB1 (budding uninhibited by benzimidazole 1), one of the earliest SAC proteins to target to the kinetochore, is recruited to the kinetochore during prophase (Jablonski, Chan et al. 1998). KNL1

is essential for the kinetochore localization of BUB1 and BUBR1 (budding uninhibited by benzimidazole-related 1) (Desai, Rybina et al. 2003, Shepperd, Meadows et al. 2012, Yamagishi, Yang et al. 2012), and BUB1 is essential for the recruitment of most downstream SAC proteins, including MAD1 (mitotic arrest deficient 1) and the members of the mitotic checkpoint complex (MCC) (Sharp-Baker and Chen 2001, Johnson, Scott et al. 2004).

The MCC is highly conserved among species and is comprised of MAD2, BUBR1, BUB3, and CDC20 (cell division cycle 20) (Sudakin, Chan et al. 2001). Upon their recruitment to the kinetochore, the members of the MCC assemble in a stepwise fashion (Musacchio and Salmon 2007). First, MAD2 binds to CDC20 via the MAD2 template reaction, which is described below (De Antoni, Pearson et al. 2005). Then, BUB3 and BUBR1, which can be found binding to each other throughout the cell cycle, are recruited by MAD2 to the MCC (Hardwick, Johnston et al. 2000, Chen 2002, Musacchio and Salmon 2007). The MCC acts as the key effector of SAC activity through its interactions with the anaphase promoting complex/cyclosome (APC/C). Schematic drawings of the MCC and its interactions with APC/C appear in Figure 1-5.

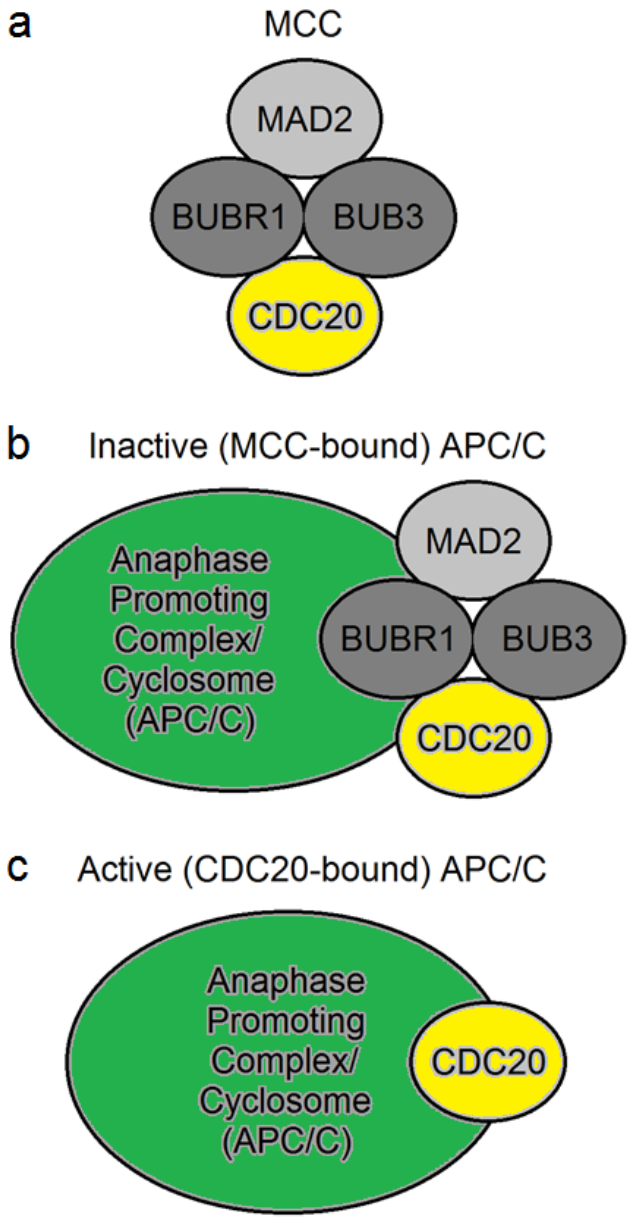


Figure 1-5. The mitotic checkpoint complex (MCC) and its role in maintaining APC/C in an inactive state. a) MCC: The mitotic checkpoint complex (MCC) includes MAD2, BUBR1, BUB3, and CDC20. Members of the MCC are recruited to unattached kinetochores during the early phases of mitosis and are the key downstream effectors of the mitotic SAC. **b) Inactive (MCC-bound) APC/C:** When the SAC is active, the MCC binds to the anaphase promoting complex/cyclosome (APC/C) at the kinetochore, maintaining APC/C in an inactive state. **c) Active (CDC20-bound) APC/C:** When the SAC is satisfied by complete formation of kinetochore-spindle attachments, the MCC dissociates, allowing CDC20 to bind to a different subunit of APC/C and activate it.

The MAD2 template reaction is a MAD1-dependent process which is necessary for formation of the MCC (De Antoni, Pearson et al. 2005, Maldonado and Kapoor 2011). MAD2 exists in two conformations, open MAD2 (O-MAD2) and closed MAD2 (C-MAD2). O-MAD2 is the free, unbound form (Luo, Tang et al. 2004). MAD1 recruits O-MAD2 to unattached kinetochores and induces a conformational change in MAD2 to the closed form (De Antoni, Pearson et al. 2005, Yu 2006). Together they form the MAD1-C-MAD2 core complex (Luo, Tang et al. 2002, Sironi, Mapelli et al. 2002). C-MAD2 which is bound to MAD1 can bind O-MAD2 and catalyze its binding to CDC20. When O-MAD2 binds to CDC20, it is released from the MAD1-C-MAD2 core complex and changes to the closed conformation. C-MAD2-CDC20 is formed, and the MAD1-C-MAD2 core complex is free to catalyze the production of additional C-MAD2-CDC20 (Mapelli, Massimiliano et al. 2007, Lad, Lichtsteiner et al. 2009). Finally, when kinetochore-spindle attachment satisfies the SAC, the MAD1-C-MAD2 core complex is stripped from the kinetochore and the production of C-MAD2-CDC20 is no longer catalyzed (Chen, Waters et al. 1996, Li and Benezra 1996, Lara-Gonzalez, Westhorpe et al. 2012). A schematic depicting the MAD2 template reaction appears in Figure 1-6.

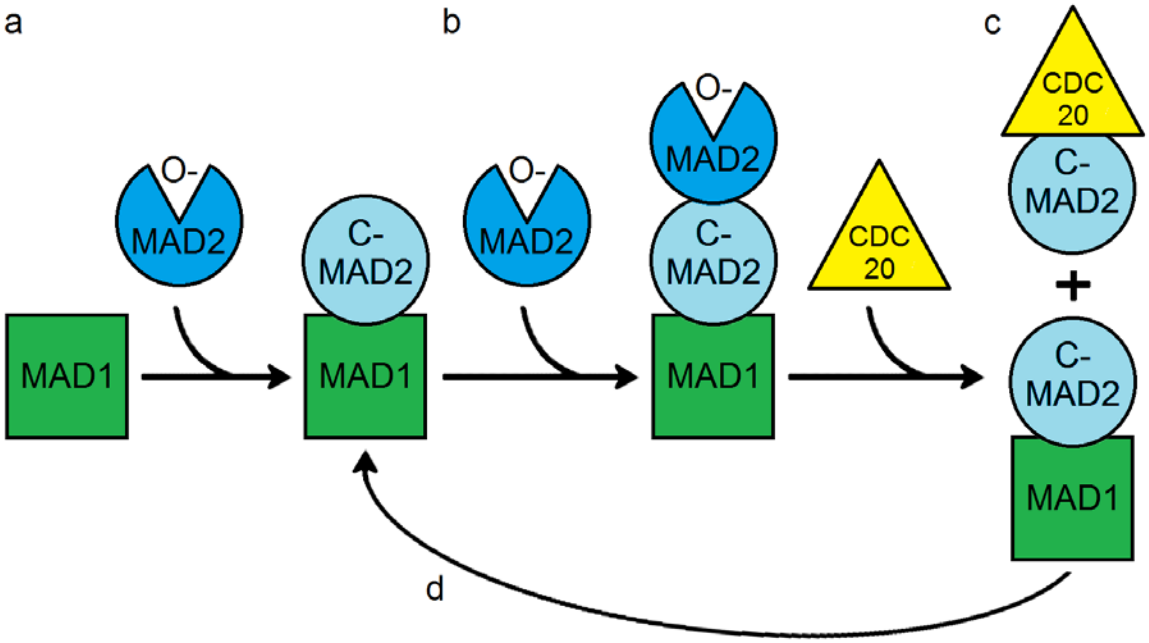


Figure 1-6. In the MAD2 template reaction, MAD1 recruits MAD2 and catalyzes the generation of MAD2-CDC20. a) MAD1 recruits O-MAD2 and catalyzes a change in conformation of O-MAD2 to C-MAD2. Together, MAD1 and C-MAD2 form the MAD1-C-MAD2 core complex. **b)** The MAD1-C-MAD2 core complex recruits additional O-MAD2, and catalyzes its binding to CDC20. **c)** C-MAD2-CDC20 is produced. **d)** The MAD1-C-MAD2 core complex is then free to catalyze the production of additional C-MAD2-CDC20.

The MCC prevents anaphase initiation by binding to the anaphase promoting complex/cyclosome (APC/C) and maintaining it in an inactive state (Li, Gorbea et al. 1997, Jablonski, Chan et al. 1998, Sudakin, Chan et al. 2001, Tang, Bharadwaj et al. 2001, Fang 2002). Satisfaction of the SAC occurs when the last unattached kinetochore becomes properly attached to the mitotic spindle (Rieder, Cole et al. 1995). At this point, the MCC dissociates, APC/C is activated, and anaphase is initiated (D'Angiolella, Mari et al. 2003, Jia, Li et al. 2011).

The anaphase promoting complex/cyclosome (APC/C) is a multisubunit E3 ubiquitin ligase which governs the metaphase-anaphase transition (Sudakin, Ganoth et al. 1995, Peters, King et al. 1996, Zachariae, Shin et al. 1996, Li, Gorbea et al. 1997, Yamada, Kumada et al. 1997). APC/C co-localizes with members of the MCC at the kinetochore, and the activity of the SAC is required for the recruitment of APC/C to this location (Acquaviva, Herzog et al. 2004). APC/C which is bound to the MCC is inactive (Li, Gorbea et al. 1997, Fang, Yu et al. 1998, Sudakin, Chan et al. 2001, Tang, Bharadwaj et al. 2001, Fang 2002). The classical idea that APC/C is activated by binding to CDC20 has been refined by studies which show that CDC20 is actually bound to inactive APC/C as part of the MCC (Fang, Yu et al. 1998, Sudakin, Chan et al. 2001). However, when the SAC is silenced and the MCC dissociates, CDC20 binds to a different subunit of APC/C and activates it (Izawa and Pines 2011). As previously noted, a diagram of the MCC and its interactions with APC/C was shown in Figure 1-5.

The activated APC/C(CDC20) has E3 ubiquitin ligase activity toward cyclin B and securin (Sudakin, Ganoth et al. 1995, Yu, King et al. 1996, Yamano, Tsurumi et al. 1998, Hershko 1999, Hagting, Den Elzen et al. 2002). Proteosomal degradation of cyclin B and securin allows the dividing cell to enter anaphase (Hagting, Den Elzen et al. 2002). Cyclin B and securin are important targets of APC/C at the metaphase-to-anaphase transition because Cyclin B plays key roles in mitotic entry and progression (Lindqvist, van Zon et al. 2007), and securin is essential for the maintenance of sister chromatid cohesion prior to anaphase (Mehta, Rizvi et al. 2012a).

Cyclin B is responsible for mitotic progression. During G2, Cyclin B is bound to CDK1 (cyclin-dependent kinase 1), but the CDK1(cyclin B) complex is inactive (Gavet and Pines 2010). Progressive activation of the CDK1(cyclin B) complex by the CDC25 phosphatase family leads to mitotic entry (Nilsson and Hoffmann 2000). Activated CDK1(cyclin B) initiates prophase by phosphorylating nuclear lamins and condensin, resulting in nuclear envelope breakdown and chromosome condensation, respectively (Peter, Nakagawa et al. 1990, Enoch, Peter et al. 1991, Luscher, Brizuela et al. 1991, Kimura and Hirano 2000, Abe, Nagasaka et al. 2011, Mall, Walter et al. 2012). When cyclin B is degraded following ubiquitination by APC/C, free CDK1 phosphorylates substrates involved in anaphase initiation and mitotic exit (Ubersax, Woodbury et al. 2003).

Securin is part of a mechanism for ensuring genomic integrity (Mehta, Rizvi et al. 2012b). To guarantee that each daughter cell receives a full complement of chromosomes, each sister chromatid must be coupled to its

partner from the time of S-phase DNA replication through the onset of anaphase (Tanaka, Fuchs et al. 2000). This coupling is achieved by the cohesin complex, which includes the proteins SCC1 (sister chromatid cohesion 1), SCC3, SMC1 (structural maintenance of chromosomes 1), and SMC3 (Michaelis, Ciosk et al. 1997, Panizza, Tanaka et al. 2000). The cohesin complex is believed to be a ring-shaped structure which encircles the sister chromatid pair (Gruber, Haering et al. 2003). While cohesin complexes run the entire length of the sister chromatid pair, they are most concentrated at the highly condensed centromere region, resulting in the tightest binding between sister chromatids at the centromere (Tanaka, Cosma et al. 1999, Weber, Gerton et al. 2004).

The protein separase has the ability to enzymatically cleave the SCC1 protein subunit of the cohesin complex (Uhlmann, Lottspeich et al. 1999, Waizenegger, Hauf et al. 2000, Nakajima, Kumada et al. 2007). However, securin sequesters separase and prevents it from cleaving SCC1. When securin is degraded following ubiquitination by APC/C, separase is released. Then, separase cleaves the cohesin complexes which link the two members of each sister chromatid pair (Ciosk, Zachariae et al. 1998, Zou, McGarry et al. 1999). The cleavage of cohesin separates each sister chromatid from its partner and enables migration of sister chromatids to opposing spindle poles when the spindle microtubules shorten during anaphase (Tanaka, Fuchs et al. 2000).

After APC/C triggers anaphase entry by ubiquitinating cyclin B and securin, the SAC is silenced and completion of cell division occurs. If the SAC is regulated properly, the result will be two euploid daughter cells. If the SAC is not

regulated properly, chromosome mis-segregation may occur, leading to the development of aneuploidy and cancer (Bharadwaj and Yu 2004).

The mitotic SAC, aneuploidy, and cancer

The mitotic SAC is a tumor suppressor signaling pathway that prevents aneuploidy by ensuring proper chromosome segregation. In the event that one or more unattached kinetochores are present in a dividing cell, the SAC will delay chromosome segregation until all kinetochore-spindle attachments have formed (Meraldi, Draviam et al. 2004). For the SAC to function properly, each SAC component must be expressed at the appropriate level. When the activity of a particular SAC effector is lost due to mutation, altered transcriptional regulation, or epigenetic modification, the signal responsible for maintaining the SAC may be weakened. A weakened SAC response may signal metaphase arrest in response to multiple unattached kinetochores, but fail to maintain the checkpoint when only a single unattached kinetochore (or just a few unattached kinetochores) remains (Rieder and Maiato 2004, Kops, Weaver et al. 2005). Thus, a weakened SAC may directly lead to aneuploidy through missegregation of chromosomes (Bharadwaj and Yu 2004). The mechanisms by which the mitotic SAC ensures genomic integrity and by which weakened SAC activity may lead to the development of aneuploidy are illustrated in Figure 1-7.

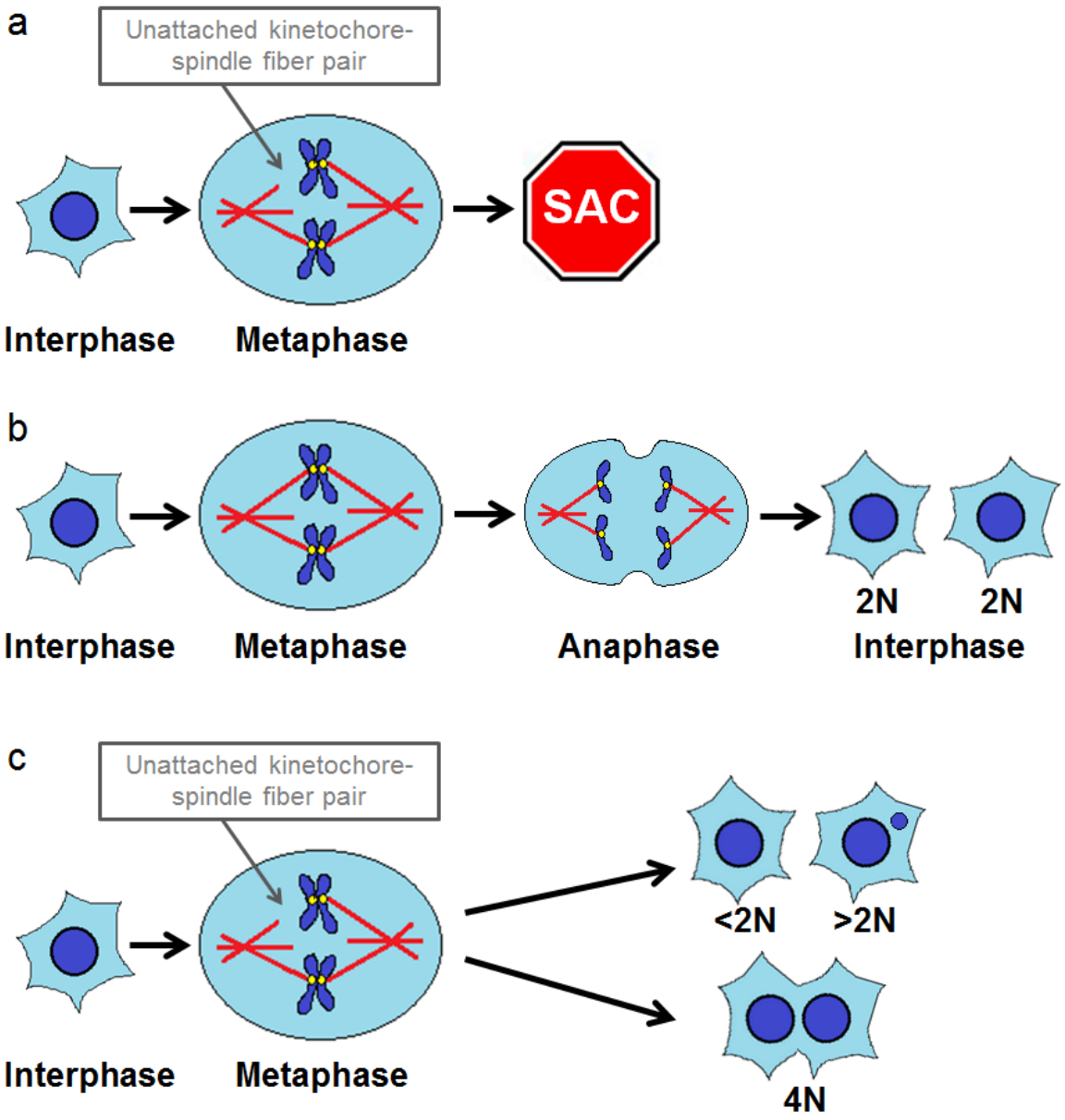

a
Unattached kinetochore-spindle fiber pair
SAC
Interphase
Metaphase
b
Interphase
Metaphase
Anaphase
2N
2N
Interphase
c
Unattached kinetochore-spindle fiber pair
Interphase
Metaphase
<2N
>2N
4N

Figure 1-7. The mitotic SAC is the key cell cycle checkpoint that regulates the metaphase-to-anaphase transition in order to protect genomic integrity. **a)** The mitotic SAC monitors spindle fiber-kinetochore attachment and is activated in the presence of one or more unattached kinetochores. **b)** The mitotic SAC delays the onset of anaphase until the mitotic spindle has properly attached to individual sister chromatids. At this point, the SAC is inactivated and the dividing cell is allowed to enter anaphase, resulting in two euploid daughter cells. **c)** Normally, the SAC is active when an unattached kinetochore-spindle fiber pair is present (see **a**). However, a dividing cell with weakened mitotic SAC activity may complete mitosis in the presence of unattached kinetochores, resulting in aneuploidy. A single sister chromatid may travel to the wrong daughter cell as a lagging chromosome and become a micronucleus (shown above) or the cell may fail to complete cytokinesis subsequent to SAC failure and become multinucleated (shown below). The presence of micronucleation and multinucleation have been observed in FA-deficient primary cells.

Aneuploidy is considered a hallmark of cancer (Gordon, Resio et al. 2012). The accumulation of multiple genetic alterations is required to promote the development of cancer, and the formation of aneuploidy in a cell with weakened SAC activity is likely to facilitate the development of cancer as losses and gains of whole and partial chromosomes result from defective chromosome segregation (Hanahan and Weinberg 2011). Chromosomal gains may result in the accumulation of additional copies of oncogenes, while chromosomal losses may lead to loss of heterozygosity for tumor suppressor genes (Lopes and Sunkel 2003). While weakened activity of the mitotic SAC is clearly linked to the development of aneuploidy, the link to tumorigenesis is not as definitively established (Silva, Barbosa et al. 2011). However, the evidence supports the idea that chromosomal instability due to a weakened SAC contributes to cancer predisposition (Bharadwaj and Yu 2004, Kops, Weaver et al. 2005, Qi and Yu 2006).

Mutations of known SAC components have been identified in a number of aneuploid cancers. In aneuploid colon cancers, mutations in SAC genes are frequently detected, including mutations in the genes encoding BUB1, BUBR1, ZW10 (Zeste White 10), Zwilch, and Rod (Cahill, Lengauer et al. 1998, Wang, Cummins et al. 2004). Mutations in the genes encoding BUBR1 and MAD1 have been detected in lymphoma (Ohshima, Haraoka et al. 2000, Tsukasaki, Miller et al. 2001). Additionally, mutations in MAD1 and MAD2 have been detected in breast cancers (Percy, Myrie et al. 2000, Tsukasaki, Miller et al. 2001). Importantly, MAD2 is under the transcriptional control of BRCA1 and mutations in

BRCA1 directly result in decreased expression of MAD2 resulting in SAC impairment (Wang, Yu et al. 2004). Mutations in BUB1 have also been found in lung cancer and pancreatic cancer, and mutations in the gene encoding MAD1 have been found in lung cancer and prostate cancer (Nomoto, Haruki et al. 1999, Gemma, Seike et al. 2000, Tsukasaki, Miller et al. 2001, Hempen, Kurpad et al. 2003).

Somatic mutations of SAC genes may be either a causal event in the development of cancer or a secondary event (Qi and Yu 2006). The first direct evidence that aneuploidy resulting from a weakened SAC plays a causal role in the development of cancer came from studies of a rare autosomal recessive genetic disease (Hanks, Coleman et al. 2004, Qi and Yu 2006). Generally, patients with mosaic variegated aneuploidy syndrome have aneuploidy in at least one fourth of the cells in multiple tissue types. In a study which attempted to identify causal mutations for mosaic variegated aneuploidy syndrome, biallelic mutations in the gene encoding the SAC protein BUBR1 were found in five of eight patients studied. Two of the five patients in the study developed embryonic rhabdomyosarcomas in different tissues as children, indicating that aneuploidy due to a weakened SAC may indeed play a causal role in the development of cancer (Hanks, Coleman et al. 2004).

The FA signaling network and mitosis

A number of studies provide hints that the FA signaling network may protect genomic integrity by playing novel roles in mitosis. Many proteins which participate in the FA/BRCA DNA repair pathway have known roles in mitosis (Wang, Yu et al. 2004, Chan, North et al. 2007, Lee, Hwang et al. 2010, Stolz, Ertych et al. 2010a, Stolz, Ertych et al. 2010b). Biochemistry studies have identified the presence of an FA core complex (CC) of unique size and subcellular location during mitosis (Thomashevski, High et al. 2004). A link between the FA pathway and the mitotic master regulator CDK1 has been established (Kruyt, Dijkmans et al. 1997, Kupfer, Yamashita et al. 1997, Mi, Qiao et al. 2004, Thomashevski, High et al. 2004). Physical interaction screens have identified biochemical interactions between FA proteins and mitotic regulators, and in some cases functional significance was ascribed to these interactions (Thomashevski, High et al. 2004, Du, Chen et al. 2009, Kim, Hwang et al. 2013). Notably, recent studies have begun to elucidate a connection between specific members of the FA signaling network and centrosome biology (Kim, Hwang et al. 2013, Zou, Tian et al. 2013). Taken together, these findings suggest potential involvement of the FA pathway in mitotic regulation.

Many proteins that participate with the FA pathway in the repair of DNA crosslink damage have also been implicated in mitosis. The E3 ubiquitin ligase BRCA1 (breast cancer susceptibility 1) is a known regulator of the mitotic SAC and is essential for proper chromosome segregation (Wang, Yu et al. 2004, Bae, Rih et al. 2005, Chabalier, Lamare et al. 2006). The essential role of BRCA1 at

the mitotic SAC may be due to its role as a transcriptional regulator of the MCC protein MAD2. BRCA1 upregulates expression of MAD2 by binding its promoter (Wang, Yu et al. 2004, Qi and Yu 2006). During mitosis, BRCA1 localizes to the mitotic spindle, spindle-kinetochore interface, midbody, and centrosome (Lotti, Ottini et al. 2002). BRCA1 additionally plays an essential role in centrosome maintenance. RNAi knockdown of BRCA1 results in centrosome amplification and fragmentation. At the centrosome, BRCA1 interacts with gamma-tubulin and ubiquitinates gamma-tubulin at lysines 48 and 344 (K48 and K344). Furthermore, through generation of a construct expressing the genetic mutant γ-tubulin K48R, it has been shown that BRCA1-mediated ubiquitination of gamma-tubulin at K48 is essential for centrosome maintenance (Xu, Weaver et al. 1999, Starita, Machida et al. 2004, Sankaran, Starita et al. 2005, Ko, Murata et al. 2006, Parvin and Sankaran 2006, Sankaran and Parvin 2006, Sankaran, Starita et al. 2006).

In addition to its roles in the regulation of the mitotic SAC and centrosome maintenance, BRCA1 regulates formation of the mitotic spindle in conjunction with CHK2 (checkpoint kinase 2). CHK2 and BRCA1 are tumor suppressors whose roles in DNA damage repair and genomic instability are well established. CHK2 phosphorylates BRCA1 at serine 988 following damage to microtubules during mitosis (Chabalier-Taste, Racca et al. 2008). Building on these findings, another research group discovered that BRCA1 and CHK2 functionally interact in the regulation of spindle assembly and genomic stability. This group found that “normal spindle assembly and mitotic progression require the Chk2-mediated phosphorylation of Ser 988 on Brca1” (Stolz, Ertych et al. 2010b) and later

summarized their findings as follows.

> "Recently, we have identified CHK2 and BRCA1 as genes required for the maintenance of chromosomal stability and have shown that a Chk2-mediated phosphorylation of Brca1 is required for the proper and timely assembly of mitotic spindles. Loss of CHK2, BRCA1 or inhibition of its Chk2-mediated phosphorylation [at serine 988 during mitosis] inevitably results in the transient formation of abnormal spindles that facilitate the establishment of faulty microtubule-kinetochore attachments associated with the generation of lagging chromosomes" (Stolz, Ertych et al. 2010a).

CHK2 is activated by FANCO/RAD51C in the DNA damage response (Somyajit, Subramanya et al. 2010). Thus, CHK2 and BRCA1 are functionally linked to the FA pathway in DNA damage repair and also have known roles in mitosis.

Two additional proteins that functionally interact with the FA pathway in DNA damage repair and have known roles in mitosis are the serine/threonine kinases ATM and ATR. ATM and ATR act as DNA damage sensors and activate the appropriate DNA repair pathways. ATM and ATR phosphorylate several proteins in the FA/BRCA pathway including FANCA, FANCM, FANCD2, FANCI, FANCJ/BACH1/BRIP1, and BRCA1 (Kim, Lim et al. 1999, Chen 2000, Tibbetts, Cortez et al. 2000, Gatei, Zhou et al. 2001, Taniguchi, Garcia-Higuera et al. 2002, Pichierri and Rosselli 2004, Ho, Margossian et al. 2006, Collins, Wilson et al. 2009, Sobeck, Stone et al. 2009, Castillo, Bogliolo et al. 2011, Sakasai, Sakai et al. 2012, Sareen, Chaudhury et al. 2012, Shigechi, Tomida et al. 2012, Singh, Ali et al. 2013, Tomida, Itaya et al. 2013). They also regulate mitotic entry. When ATM and ATR are activated in response to DNA double-strand breaks or uncapped telomeres, they phosphorylate p53, CHK1, and CHK2, leading to cell cycle arrest prior to mitosis and prevention of spindle assembly (Brown and

Costanzo 2009, Smith, Dejsuphong et al. 2009, David 2012, Thanasoula, Escandell et al. 2012). ATM has also been shown to play a role in cell cycle arrest in response to DNA damage which occurs during mitosis. In DNA-damaged mitotic cells, ATM and CHK1 were found to be essential for PP2A (protein phosphatase 2A)-mediated dephosphorylation of PLK1 (polo-like kinase 1) and subsequent cell cycle arrest (Lee, Hwang et al. 2010).

The DNA helicase BLM is the final FA pathway interactant with mitotic roles which will be discussed. BLM is known to participate in anaphase sister chromatid decatenation and subsequently localize to structures termed ultrafine bridges. Ultrafine bridges are BLM-coated structures which were described to link sister chromatids at sites of fragile and broken chromatin during anaphase and telophase (Chan, North et al. 2007). Recent studies have suggested that the FA pathway may collaborate with BLM in its mitotic roles. FANCD2 and FANCI were discovered to localize to the termini of BLM-coated ultrafine bridges, and FANCD2 was shown to be essential for the recruitment of BLM to these structures (Chan, Palmai-Pallag et al. 2009, Naim and Rosselli 2009). Another study provides a hint that the FA pathway may itself regulate cell division. This study showed that FA-deficient murine and human primary cells have increased rates of ultrafine bridges and binucleation. Utilizing spontaneous binucleation as an endpoint, the study concluded that loss of an individual FA protein results in cytokinesis failure in murine and human cells (Vinciguerra, Godinho et al. 2010).

Biochemistry-based studies examining the localization and regulation of FA proteins provide further hints that the FA pathway may play a unique role

during mitosis. It has been established that the FA CC exists during interphase in both cytoplasmic and nuclear forms. However, the FA CC departs from the nucleus at the onset of mitosis and exists in the cytoplasm in a uniquely-sized 750-kDa form (Qiao, Moss et al. 2001, Thomashevski, High et al. 2004). Phosphorylation of FANCG was observed at the onset of mitosis and was shown to temporally correlate with the departure of the FA CC from chromatin (Qiao, Moss et al. 2001). Nuclear export sequences have been identified on FANCA and may be responsible for the nuclear exit of the FA CC at the onset of mitosis (Ferrer, Rodriguez et al. 2005). Another study found that FANCM is hyper-phosphorylated at the onset of mitosis, leading to its dissociation from the rest of the FA CC and its subsequent proteosomal degradation (Kee, Kim et al. 2009).

A link between the FA pathway and the mitotic master regulator CDK1 has been established. FANCA, FANCC, and FANCG physically interact with CDK1 (Kupfer, Yamashita et al. 1997, Thomashevski, High et al. 2004). FANCC functions in an upstream regulatory pathway controlling CDK1 activity (Kruyt, Dijkmans et al. 1997). Furthermore, CDK1 phosphorylates FANCG at serine 387 at the onset of mitosis (Mi, Qiao et al. 2004). A model is presented in Figure 1-8 which incorporates the biochemistry-based studies from the last two paragraphs.

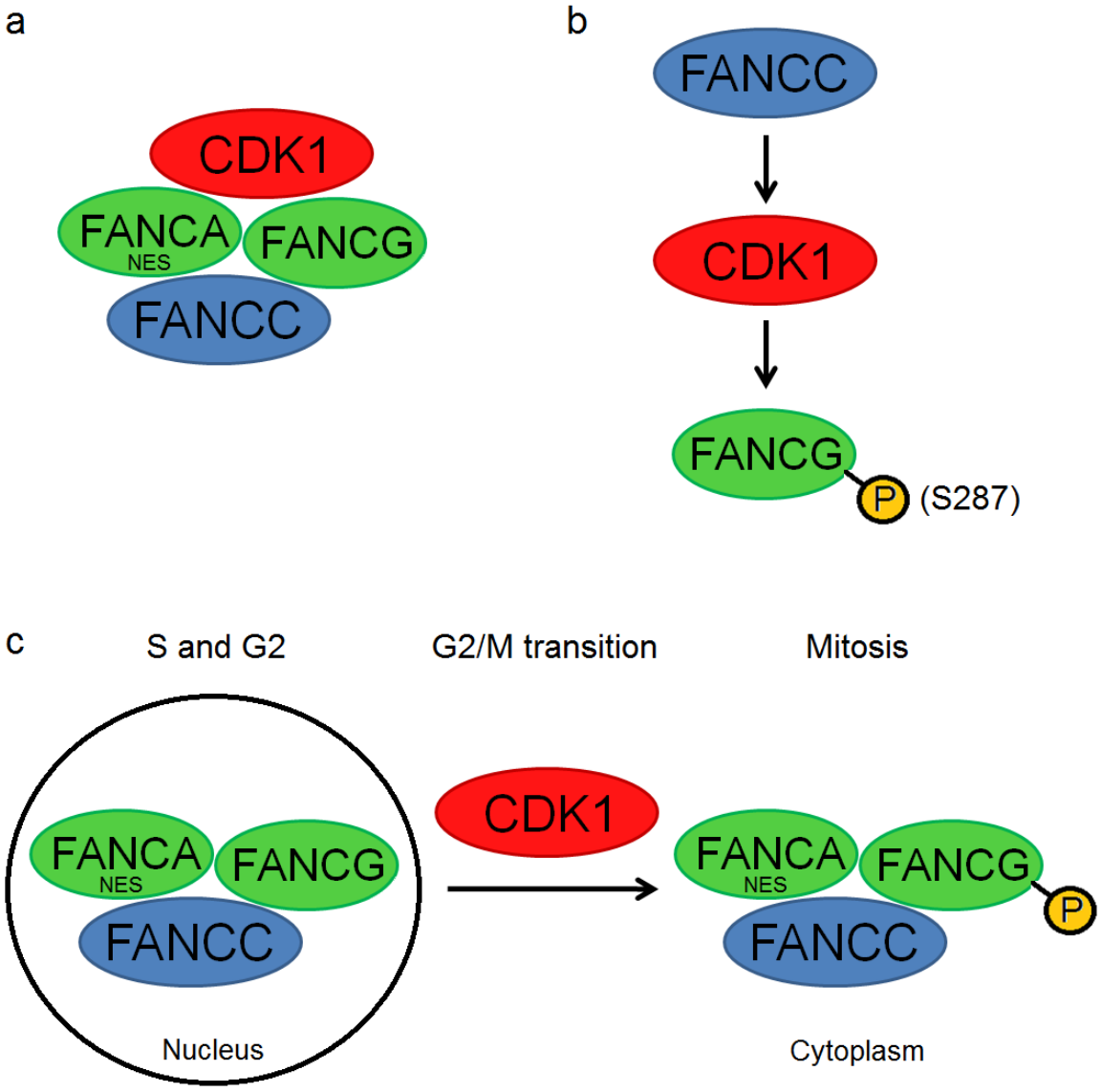


Figure 1-8. Proposed model for the interaction of CDK1 with the FA core complex at the onset of mitosis. a) The FA core complex (CC) members FANCA, FANCC, and FANCG physically interact with the mitotic cyclin-dependent kinase CDK1. **b)** FANCC functions in an upstream pathway which activates CDK1, and CDK1 phosphorylates FANCG at serine 287 at the onset of mitosis. **c)** Phosphorylation of FANCG at serine 287 temporally correlates with departure of the FA CC from chromatin. Nuclear export sequences in FANCA may mediate the redistribution of the FA CC from the nucleus to the cytoplasm at the onset of mitosis.

A number of physical interaction screens have identified binding partners of FANCA. Of specific interest to my project, it has been shown that FANCA physically interacts with a number of known mitotic regulators. BRCA1 and FANCA interact independently of the DNA damage response (Folias, Matkovic et al. 2002). (As previously noted, BRCA1 participates with the FA signaling network in the repair of interphase DNA damage and also regulates the mitotic SAC.) FANCA also interacts with the SAC regulator CENPE (centromere protein E) (Du, Chen et al. 2009). CENPE is a kinetochore-localized kinesin which physically interacts with the MCC protein BUBR1 and induces auto-phosphorylation of BUBR1 when the kinetochore is unattached to the mitotic spindle (Guo, Kim et al. 2012). Because BUBR1's autophosphorylation is essential for BUBR1's SAC activity, CENPE is an essential regulator of the mitotic SAC (Tanudji, Shoemaker et al. 2004). When kinetochore-spindle attachment satisfies the SAC, CENPE binds to microtubules and silences BUBR1's kinase activity (Mao, Desai et al. 2005).

Recently, it was shown that FANCA physically interacts with the centrosomal protein γtubulin and the kinetochore- and centrosome-associated protein NEK2 (NIMA [never-in-mitosis-gene A]-related kinase 2) (Kim, Hwang et al. 2013). Gamma-tubulin plays an important role in microtubule nucleation throughout the cell cycle and is essential for the assembly of the mitotic spindle (Stearns and Kirschner 1994, Moritz, Braunfeld et al. 1995, Shu and Joshi 1995, Zimmerman, Sillibourne et al. 2004). NEK2 is a serine/threonine kinase which is essential for centrosome maintenance (Fry, Meraldi et al. 1998, Uto and Sagata

2000), bipolar spindle assembly (Faragher and Fry 2003), proper chromosome congression (Fu, Ding et al. 2007), localization of MAD2 to the kinetochore (Lou, Yao et al. 2004), faithful kinetochore-spindle attachment (Du, Cai et al. 2008), SAC arrest (Lou, Yao et al. 2004), and accurate chromosome segregation (Lou, Yao et al. 2004, Sonn, Khang et al. 2004). NEK2 is one of the targets of activated APC/C(CDC20) at the metaphase-anaphase transition, when APC/C(CDC20) shuts down the SAC (Hames, Wattam et al. 2001). In summary, FANCA physically interacts with several known regulators of centrosome biology, spindle dynamics, and the mitotic SAC.

A recent study found that FANCJ physically interacts with the mitotic regulator PLK1 (polo-like kinase 1) (Zou, Tian et al. 2013). PLK1 is a serine/threonine kinase that controls progression through mitosis and cytokinesis and has a diverse range of known functions. At the transition from interphase into mitosis, PLK1 plays important roles in checkpoint recovery (shutting down the DNA damage response in order to prepare the cell for the transition from G2 into mitosis), centrosome maturation, and mitotic entry. Furthermore, PLK1 controls the execution of cell division by regulating spindle assembly, sister chromatid separation, and cytokinesis (Bruinsma, Raaijmakers et al. 2012). The significance of FANCA's physical interaction with NEK2 and FANCJ's physical interaction with PLK1 will be discussed in the following paragraph.

Recent studies have begun to elucidate a functional connection between the FA pathway and centrosome biology. One study established a role for FANCA in centrosome maintenance (Kim, Hwang et al. 2013). This study

established that FANCA localizes to the centrosome and physically interacts with the known centrosomal proteins NEK2 and γtubulin. In RNAi-knockdown cells and FA patient fibroblasts, loss of FANCA resulted in centrosome amplification. Furthermore, the study demonstrated that NEK2 phosphorylates FANCA at T351 and that phosphorylation of FANCA at this site is essential for centrosome maintenance (Kim, Hwang et al. 2013).

Another study examined the role of FANCJ in centrosome maintenance in U2-OS cells (Zou, Tian et al. 2013). FANCJ localizes to centrosomes and RNAi knockdown of FANCJ results in centrosome amplification (~two-fold increase in the fraction of cells with >2 centrosomes in FANCJ siRNA-transfected cells compared with negative control). These results indicate that FANCJ is essential for centrosome maintenance. Treatment of U2-OS cells with MMC results in a marked amplification of centrosomes (~10-fold increase in the fraction of cells with >2 centrosomes in 0.5-1 uM MMC-treated cells compared with untreated cells). Additionally, treatment with the DNA interstrand crosslinking agent MMC promotes FANCJ's localization to centrosomes, and RNAi knockdown of FANCJ partially rescues cells from MMC-induced centrosome amplification. Thus, the authors of the study concluded that FANCJ mediates MMC-induced centrosome amplification. Furthermore, FANCJ physically interacts with PLK1, inhibition of PLK1 partially rescues MMC-induced centrosome amplification, and PLK1 and FANCJ have an epistatic functional relationship in MMC-induced centrosome amplification. Finally, RNAi knockdown of FANCJ no longer decreases the MMC-induced centrosome amplification in cells expressing a PLK1 mutant that has

constitutive kinase activity. Thus, the authors of the study concluded that FANCJ mediates MMC-induced centrosome amplification through activation of PLK1. The same study found that FANCJ and five other FA proteins (FANCA, FANCB, FANCG, FANCI, and FANCM) localize to the centrosome (Zou, Tian et al. 2013).

In summary, both FANCA and FANCJ localize to the centrosome, physically interact with known centrosomal proteins, and are essential for centrosome maintenance. Furthermore, NEK2-mediated phosphorylation of FANCA is required for FANCA's essential role in centrosome maintenance, and FANCJ and PLK1 functionally interact to effect MMC-induced centrosome amplification (Kim, Hwang et al. 2013, Zou, Tian et al. 2013).

Summary and Significance

The sixteen members of the FA signaling pathway act as tumor suppressors by functioning in the maintenance of genomic integrity. The heterogenous genetic disease FA results from biallelic germline mutations in any one of the known FA genes (Taniguchi 2009, D'Andrea 2010). FA patients have a high predisposition to cancer, especially AML, MDS, and SCC (Alter, Giri et al. 2010). Moreover, somatic alterations in the FA genes have been implicated in a significant proportion of pancreatic, breast, and ovarian cancers arising in the general population (Schutte, da Costa et al. 1995, van der Heijden, Yeo et al. 2003, Jones, Hruban et al. 2009).

Genomic instability in FA-deficient cells is characterized by impaired DNA-damage repair, chromosome breaks, and gross aneuploidy (Berger, Bernheim et al. 1980b, Berger, Le Coniat et al. 1993, Tutt, Gabriel et al. 1999, D'Andrea 2003, van der Heijden, Yeo et al. 2003, Mehta, Harris et al. 2010). It has been known for decades that FA-deficient cells have a high frequency of aneuploidy and micronucleation (Berger, Bernheim et al. 1980b, Willingale-Theune, Schweiger et al. 1989, Berger, Le Coniat et al. 1993, Maluf and Erdtmann 2001, Mehta, Harris et al. 2010), but it is not fully understood how disruption of the FA signaling pathway leads to these gross chromosomal abnormalities. The role of the FA signaling cascade in interphase DNA-damage repair is well-established (Grompe and D'Andrea 2001, Bagby and Alter 2006, Williams, Wilson et al. 2011), but the molecular basis for the development of aneuploidy in FA-deficient cells is incompletely understood. Our previous work confirmed the correlation between

gross aneuploidy, myelodysplasia, and leukemia in the *Fancc-/-; Fancg-/-* mouse, which is the first murine model to recapitulate the hematopoietic manifestations of the human disease (Pulliam-Leath, Ciccone et al. 2010). In BM cells from *Fancc-/-; Fancg-/-* mice, dysregulation of the known mitotic spindle checkpoint regulators Mad2 and BubR1 was observed in a gene chip-based genome-wide transcriptomal assay (A. Pulliam-Leath, S. Ciccone, G. Nalepa, G. Bagby, D. W. Clapp, unpublished data).

These results led us to hypothesize that the aneuploidy and oncogenesis observed in FA may result from defective regulation of mitosis in FA-deficient cells. In particular, we hypothesized that the human FA proteins may be essential for the mitotic SAC. The mitotic SAC is a tumor suppressor pathway that prevents aneuploidy by ensuring accurate chromosome segregation during cell division (Kops, Weaver et al. 2005, Musacchio and Salmon 2007, Gordon, Resio et al. 2012). Thus, we reasoned that a weakened SAC could provide an explanation for aneuploidy and micronucleation in FA-deficient cells.

Data presented here establish that the FA signaling network is essential for mitotic SAC activity and centrosome maintenance, demonstrate that FA proteins localize to the mitotic spindle and centrosomes in a cell cycle-dependent manner, and elucidate the role of the FANCA protein in the activity of the mitotic SAC. These findings implicate the FA pathway in mitosis and offer an explanation for the aneuploidy and oncogenesis that result from inactivation of the FA pathway.

CHAPTER TWO

MATERIALS AND METHODS

Cell culture

HeLa cells were cultured in high glucose-containing Dulbecco's modified eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Primary human skin fibroblasts from FA patients and healthy controls were cultured at 5% oxygen in high glucose-containing DMEM supplemented with 10% FBS, 1% glutamine, 1% sodium pyruvate, and penicillin/streptomycin. Uncorrected and gene-corrected primary human skin fibroblasts derived from FANCA patients were cultured at 5% oxygen in high glucose and glutamine-containing DMEM supplemented with 10% FBS, 1% sodium pyruvate, and penicillin/streptomycin. HeLa cells stably expressing GFP-γtubulin/GFP-CENPA and GFP-H2B/mCherry-αtubulin were gifts from Claire Walczak (Indiana University, Bloomington, IN). FA patient-derived specimens used in this study were primary fibroblasts isolated from skin biopsies of FA patients and were obtained from a cryo-repository maintained by Helmut Hanenberg (Indiana University School of Medicine, Indianapolis, IN; Heinrich Heine University School of Medicine, Duesseldorf, Germany).

Study approval

Primary fibroblasts from FA patients were provided by Helmut Hanenberg (Indiana University School of Medicine, Indianapolis, IN; Heinrich Heine

University School of Medicine, Düsseldorf, Germany), who maintains a cryo-repository of FA patient specimens for research purposes (Indiana University School of Medicine, Indianapolis, IN). Helmut Hanenberg obtained the FA patient-derived cell lines used in this study from Detlev Schindler (University of Würzburg, Würzburg, Germany), Hans Joenje (VU University, Amsterdam, The Netherlands), or Arleen Auerbach (The Rockefeller University, New York, NY). Specimens derived from FA patients were obtained following approval by the appropriate local ethics committees (Ethikkommission der Universität Würzburg, Würzburg, Germany; IRB at Rockefeller University, New York, NY). The FANCF cell line obtained from Hans Joenje (VU University, Amsterdam, The Netherlands) was previously published (de Winter, Rooimans et al. 2000). Use of patient-derived specimens was subject to approval by the ethics committees of participating centers (IRB at Indiana University School of Medicine, Indianapolis, IN; Ethikkommission der Heinrich-Heine-Universität, Düsseldorf, Germany). Written informed consent was obtained from the parents of all children.

siRNA library

A customized siRNA library against all known FA proteins was designed and ordered from Ambion (Life Technologies, Foster City, CA). A validated siRNA against MAD2 was used as a positive control, and the Negative Control siRNA #1 was employed as a negative control. The siRNA screen was performed twice. The first time the screen was performed, Silencer siRNAs were ordered and the coverage was three siRNAs per gene for the thirteen known FA genes: FANCA,

FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM, FANCD2, FANCI, FANCD1 (also known as BRCA2), FANCJ (also known as BRIP1), and FANCN (also known as PALB2). The second time the screen was performed, next generation Silencer Select siRNAs were ordered for the fifteen known FA genes, the thirteen listed above plus FANCO (also known as RAD51C) and FANCP (also known as SLX4). Only one siRNA was ordered for FA proteins which were identified as potential regulators of the mitotic SAC the first time the screen was performed, while at least three siRNAs were ordered for FANCO, FANCP, and the FA proteins whose potential role at the mitotic SAC was found to be inconclusive in the first screen. The siRNAs which were utilized in the second screen were as follows: FANCA (s162, s163, s164, 120953, 105965, 7827), FANCB (s5012, s5013, s5014, s225863, s225864, 39216), FANCC (s4985), FANCE (s4992), FANCF (s5015), FANCG (s5018), FANCL (s30218), FANCM (s33619, s33620, s33621), FANCD2 (s4988), FANCI (s30461), FANCD1/BRCA2 (s2085), FANCJ/BRIP1 (s38384), FANCN/PALB2 (s36198), FANCO/RAD51C (s11737, s11738, s11739), FANCP/SLX4 (s39052, s39053, s39054), MAD2 (143483), and negative control (4390843).

siRNA screen

Reverse transfection of HeLa cells with 10 nM siRNA was performed on 96-well imaging microplates (BD Falcon) using siPORT NeoFX transfection reagent (Applied Biosystems, Carlsbad, CA). After siRNA and transfection reagent were added to each well, HeLa cells stably expressing GFP-histone H2B and

mCherry-αtubulin were plated at 2500 cells/well in BD imaging microplates. After 48 hours, the cells were exposed to 100 nM taxol for 24 hours and then fixed with 4% paraformaldehyde. Next, individual wells were imaged using a BD Pathway 855 high-throughput automated screening microscope (BD Biosystems). Using ImageJ software, manual quantification of mitotic nuclei, interphase nuclei, and multinuclei was performed in the taxol-challenged HeLa cells based on the morphology of their nuclei and microtubules.

Isolation of CD34+ human stem cells, FANCA shRNA knockdown, and phenotype characterization in ex vivo-cultured CD34+ cells

Human umbilical cord blood was obtained from the In Vivo Therapeutics Core of the Indiana University Simon Cancer Center, and CD34+ human stem and progenitor cells were isolated by magnetic-activated cell sorting (MACS). Briefly, the low density cells were separated from red blood cells by density centrifugation and by lysing the remaining red blood cells. The CD34+ cells were labeled with human CD34 antibody conjugated with magnetic microbeads (Miltenyi Biotec) and isolated at approximately 95% purity by subsequent passage through two MACS separation columns (Miltenyi Biotec). Then, human $CD34^+$ primary cells were transduced with a GFP-tagged lentiviral construct containing either scrambled negative control shRNA or a combination of two shRNA sequences against FANCA (Z. Sun and H. Hanenberg, Indiana University, Indianapolis, Indiana, USA; unpublished observations) and cultured for 4 days at 5% oxygen in Iscove's modified Dulbecco's medium supplemented

with 20% FBS, penicillin/streptomycin, 100 ng/ml stem cell factor, 100 ng/ml thrombopoietin, and 100 ng/ml FLT3 (fms-related tyrosine kinase 3). The GFP-positive cells were sorted using a FACSCalibur flow cytometer and cultured overnight as described above. The cells were pulsed with 10 μM BrdU (bromodeoxyuridine) for 4 hours and then treated with 100 nM taxol for 24 hours. Next, the cells were fixed, permeabilized, and stained with Hoechst 33342 DNA stain (Invitrogen), PE-conjugated antibody detecting phosphorylated histone H3 (Cell Signaling Technology), and APC-conjugated anti-BrdU according to the manufacturer's protocol for the BD Pharmingen APC-BrdU Kit. Finally, concurrent flow cytometry and imaging data was collected using an Amnis ImageStreamX Mark II imaging flow cytometer and was analyzed using Amnis IDEAS Application v5.0 software. Live BrdU+ G2/M cells were gated, and then phospho-histone H3+ cells and multinucleated cells were analyzed in the appropriate gated populations. To quantitate multinucleated cells, guided analysis in the IDEAS software package was used to identify cells with non-round nuclei, and then interphase cells with a single nucleus were manually subtracted.

Quantification of mitotic failure in primary cells from patients with FA

To assess mitotic SAC function in primary patient cells, human skin fibroblasts from FA patients of twelve different FA complementation groups and from healthy controls were plated at $2*10^5$ cells/well on ultrafine coverslips in 6-well plates. After approximately 16 hours, 200 nM taxol was added for 24 hours. Then, the cells were fixed in 4% paraformaldehyde. Hoechst 33342 (1 ug/mL) and Alexa

Fluor 594-labeled phalloidin (Invitrogen, Carlsbad, CA) were used to stain chromosomes and actin respectively, and then the coverslips were mounted on glass slides. A DeltaVision deconvolution microscope (Applied Precision, Issaquah, WA) equipped with a 20x objective was used for image acquisition, and ImageJ was used for quantification of cells. For flow cytometry analysis, fibroblasts from FA patients and healthy controls were plated on 6-well plates ($2*10^5$ cells/well) for approximately 16 hrs, treated with 200nM taxol for 24 hrs, and fixed in 4% paraformaldehyde. Next, cells were permeabilized in 90% methanol for 30 minutes and stained with DRAQ5 DNA stain and an Alexa Fluor 488-conjugated antibody against phospho-histone H3 (Cell Signaling) to identify the mitotic fraction. A BD FACSCalibur flow cytometer and CellQuest Pro software were used for data collection and analysis. At least three independent experiments were performed for each cell line. The specific mutations detected in the FA patient fibroblasts are listed in Table 2-1 below.

FA gene	Mutation 1	Mutation 2	Reference
FANCA	c.856 C>T (p.Q286X)	c.3976 C>T (p.Q1326X)	(Gross, Hanenberg et al. 2002)
FANCA	c.3163 C>T (p.1055W)	c.4124-4125 delCA (p.T1375fsX1423)	(Nalepa, Enzor et al. 2013)
FANCB	Dup chrX: c.14788000-14797000 (ex2+3)		(Chandrasekharappa, Lach et al. 2013)
FANCC	c.377_378 delGA, p.R126fsX127	c.377_378 delGA, p.R126fsX127	(Nalepa, Enzor et al. 2013)
FANCE	c.1111 C>T (p.R371W)	c.1111 C>T (p.R371W)	(Neveling 2007)
FANCF	c.349-395 del47	c.16C>T (p.Q6X)	(de Winter, Rooimans et al. 2000)
FANCG	c.313 G>T (p.E105X)	IVS 9+1 G>A	(Demuth, Wlodarski et al. 2000)
FANCL	c.920 G>A (p.C307Y)	c.217-20 T>G	(Nalepa, Enzor et al. 2013)
FANCD2	c.1948-16 TrG (ex22 skipping)	c. 2775_2776 CC>TT (p.R926X)	(Kalb, Neveling et al. 2007)
FANCI	c.3853 C>G (p.R1285G)	c.3853 C>G (p.R1285G)	(Scheckenbach, Wagenmann et al. 2012)
FANCD1/ BRCA2	c.706-15 del10	c.706-15 del10	(Rischewski, Hoffmann et al. 2002.)
FANCJ/ BRIP1	c.2533 C>T (p.R798X)	80037 A>T	(Levran, Attwooll et al. 2005)
FANCN/ PALB2	c.2393_2394 insCT, p.T799fs	c.3350+4 A>G, r.3350 insGCAG/ p.F1118fs	(Reid, Schindler et al. 2007)

Table 2-1. Summary of specific mutations detected in primary FA patient fibroblasts used in this project.

Genetic rescue of mitotic SAC activity in FA patient cells

Primary FANCA-deficient and FANCC-deficient patient fibroblasts from two FANCA patients and one FANCC patient were transduced with lentiviral expression constructs containing an IRES-NEO or IRES-PURO cassette (empty control vector) or a construct expressing a full-length codon-optimized *FANCA* or *FANCC* cDNA in addition to the IRES-NEO or IRES-PURO cassette. Geneticin or puromycin respectively was used to select for cells that stably expressed the vector. The result was the generation of six pairs of complementary uncorrected and gene-corrected primary FANCA and FANCC patient cells, three cell line pairs containing IRES-NEO vectors and three cell line pairs containing IRES-PURO vectors. To confirm successful genetic correction, cell cycle profiles were analyzed after treatment with MMC. The transduced cells as well as healthy control and untransduced FA patient cells were cultured in the presence of 0 nM, 33 nM, or 45 nM MMC for 4 days, permeabilized in 90% methanol, and stained with propidium iodide. A BD FACSCalibur flow cytometer and CellQuest Pro software were used for data collection and analysis. Successful genetic correction was confirmed by the ablation of G2/M block in cells transduced with the FA cDNA-containing vector. To assess the SAC in genetically corrected FANCA patient-derived cells, cells were plated on ultrafine coverslips in 6-well plates, grown to >50% confluency, and then cultured in the presence of 200 nM taxol or 100 ng/ml nocodazole for 24 hours. Hoechst 33342 (1 μg/ml) and Alexa Fluor 594-labeled phalloidin (Invitrogen) were used to stain chromosomes and actin respectively, and then the coverslips were mounted on glass slides. A

DeltaVision deconvolution microscope (Applied Precision) equipped with a ×20 objective was used for image acquisition, and ImageJ was used for manual quantification of cells.

Immunoblotting to validate siRNAs

Whole-cell extracts of HeLa cells were prepared using ProteoJET Mammalian Cell Lysis Reagent (Fermentas), followed by denaturation in SDS sample buffer. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose or methanol-activated PVDF membranes, and probed with the indicated antibodies. Membranes were developed via ECL reaction. The antibodies used include rabbit anti-FANCA (Abcam), rabbit anti-FANCB (Abcam), rabbit anti-FANCC (Abcam), rabbit anti-FANCE (Abcam), goat anti-FANCF (Abcam), mouse anti-FANCG (Abcam), rabbit anti-FANCL (Abcam), mouse anti-FANCD2 (Novus, St. Louis, MO), rabbit anti-FANCI (Abcam), rabbit anti-BRCA2/FANCD1 (Cell Signaling), mouse anti-Bach1/BRIP1/FANCJ (Invitrogen), rabbit anti-PALB2/FANCN (Abcam), mouse anti-RAD51/FANCO (Abcam), rabbit anti-SLX4/FANCP (Abcam), rabbit anti-MAD2 (Santa Cruz, Santa Cruz, CA), and mouse anti-actin (Sigma, St. Louis, MO).

Immunoblotting to rule out siRNA off-target effect on MAD2

For immunoblotting of MAD2 in siRNA-knockdown HeLa cells, HeLa cells were transfected with 10 nM siRNA for 48 hours and then whole-cell extracts were prepared by cell lysis using M-PER Mammalian Protein Extraction Reagent

(Thermo Scientific) followed by denaturation in SDS sample buffer. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and then probed with rabbit anti-MAD2 (Abcam) and mouse anti-CoxIV (Cell Signaling) or rabbit anti-GAPDH (Cell Signaling). Membranes were developed by quantitative infrared Western blot detection using a LI-COR Odyssey CLx imager.

Visualization of endogenous FA proteins during mitosis

To visualize the localization of endogenous FA proteins during mitosis, immunofluorescence microscopy was performed. HeLa cells or HeLa cells stably expressing GFP-CENPA/GFP-γtubulin were grown on ultrathin glass coverslips. Then, cells were fixed for 15 minutes in 4% paraformaldehyde and subsequently permeabilized in 0.1% Triton X-100 for 15 minutes (Sigma-Aldrich, St. Louis, MO), or they were extracted in 0.1% Triton X-100 for 2 minutes prior to 10 minute fixation in 4% paraformaldehyde. Cells which were extracted prior to fixation were further permeabilized in 0.1% Triton X-100 in PBS for 10 minutes after paraformaldehyde fixation. Cells were blocked in 1% BSA for 1-2 hours or in Image-iT FX signal enhancer for 30 minutes (Invitrogen). Then immunostaining was performed using the following antibodies: rabbit anti-BUBR1 (Cell Signaling), rabbit anti-FANCA (Abcam), rabbit anti-FANCB (Abcam), rabbit anti-FANCC (Abcam), rabbit anti-FANCD2 (Abcam), rabbit anti-FANCE (Abcam), mouse anti-FANCG (Abcam), rabbit anti-BRCA2/FANCD1 (Cell Signaling), rabbit anti-PALB2/FANCN (Abcam), mouse anti-PLK1 (Abcam), and mouse anti-αtubulin (Invitrogen). Cells were stained with primary antibodies overnight at 1:100

concentration in 1% BSA or PBS and with Alexa Fluor-conjugated fluorescent secondary antibodies (Life Technologies) for 2 hours at 1:10,000 concentration in PBS. Hoechst 33342 was used to stain DNA and Alexa Fluor-conjugated phalloidin was used to stain actin. Coverslips were mounted to ultrathin glass slides (Fisherbrand), and image acquisition was performed using a DeltaVision deconvolution microscope (Applied Precision) equipped with a 60x or 100x objective followed by 10 deconvolution cycles. All images were obtained and processed identically using softWoRx software (Applied Precision).

Generation and expression of GFP-fused FA proteins

To further visualize the localization of FA proteins during mitosis, fusion constructs between enhanced green fluorescent protein (GFP) and FANCC, FANCG, or FANCL cDNAs were generated and verified by direct sequencing (Christophe Marchal and Helmut Hanenberg, unpublished data). HeLa cells were reverse-transfected with each of the constructs using ExGene transfection reagent (Fermentas) at $1*10^5$ cells per well on coverslips in 6-well plates. Forty-eight hours post transfection, cells were fixed in 4% paraformaldehyde. Hoechst 33342 and Alexa Fluor 594-labelled phalloidin (Invitrogen) were used to stain DNA and actin respectively. Coverslips were mounted to ultrathin glass slides (Fisherbrand), and image acquisition was performed using a DeltaVision personalDV microscope (Applied Precision) equipped with a 100x objective followed by 10 deconvolution cycles. All images were obtained and processed identically using softWoRx software (Applied Precision).

Subcellular localization during mitosis

HeLa cells were treated with 9 nM RO3306 (Enzo, Farmingdale, NY). RO3306, a selective inhibitor of CDK1, was used to synchronize the cells in G2. After 24 hours, the cells were washed three times with fresh media to release the cells into mitosis. When the majority of released cells were in metaphase or anaphase by visual inspection utilizing light microscopy (approximately 2 hours later), the cells were harvested. Untreated HeLa cells were cultured under similar conditions and harvested simultaneously. Cell counting using a hemacytometer was performed for each treatment condition on a plate identical to the one used for cell lysis. Based on these cell counts, equal numbers of RO3306-treated and untreated HeLa cells were used. Whole cell extracts were prepared by cell lysis using ProteoJET Mammalian Cell Lysis Reagent (Fermentas) and then fractionated with ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's protocol. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and then probed with rabbit anti-FANCC (Abcam). Mouse anti-histone H1 (Santa Cruz) and mouse anti-vimentin (BD, Franklin Lakes, NJ) were used as fractionation controls for the nuclear and cytoskeletal fractions respectively.

Centrosome visualization and quantification

HeLa cells transfected with siRNAs as described above or human fibroblast cells from FA patients and healthy controls were fixed in 4% paraformaldehyde, permeabilized in Triton X-100 (Sigma-Aldrich), and blocked in 1% BSA.

Immunostaining was performed using a rabbit anti-pericentrin antibody (Abcam) and Alexa Fluor-conjugated fluorescent secondary antibody (Life Technologies). Hoechst 33342 was used to stain DNA and Alexa Fluor-conjugated phalloidin was used to stain actin. Coverslips were mounted to ultrathin glass slides (Fisherbrand), and image acquisition was performed using a DeltaVision deconvolution microscope (Applied Precision) equipped with a 20x objective followed by 10 deconvolution cycles. All images were obtained and processed identically using softWoRx software (Applied Precision). At least three experiments were performed for each siRNA or FA patient-derived cell line.

Hypersensitivity of FANCA-deficient cells to spindle drugs

To assess the response of FANCA-deficient cells to spindle drugs, a clonogenic assay was performed on uncorrected and gene-corrected primary FANCA patient fibroblasts. Cells were plated in 10-cm dishes at a density of 500 cells/dish and cultured overnight. Then, the media in each plate was replaced with fresh media containing 0 nM, 0.1 nM, 0.3 nM, 1 nM, or 3 nM taxol. The cells were cultured for 11 days, then stained with methylene blue. Colonies (>25 cells) were quantified by manual counting.

Preparation of cells for proteomics and phospho-proteomics screen

Uncorrected and gene-corrected primary FANCA patient fibroblasts were generated via transduction of primary FANCA patient-derived human skin fibroblasts with either empty control vector or *FANCA* cDNA-containing vector as

described above. Primary FANCA patient cells stably transduced with empty control vector (MNHN S91 IN) and the isogenic gene-corrected complement (MNHN S91 FAco IN) were cultured in 15-cm dishes for 9 days in cell culture media with or without 1 nM taxol. Ten 15-cm dishes were considered a single replicate, and the experiment was performed in triplicate. On day 9, the cells were trypsinized for 10 minutes, transferred into 50-mL conical tubes, and pelleted by centrifugation at 1550 rpm. Cells were resuspended in ice-cold PBS, and plates from the same replicate were pooled in a single tube. Each replicate was thoroughly mixed by inversion and a hemacytometer was used to quantify the number of cells. Cells were pelleted by centrifugation at 1550 rpm. Next, the cells in each tube were resuspended in 1.2 mL ice-cold PBS and transferred to an Eppendorf tube. Using a bench-top centrifuge, the cells were centrifuged at 500g for 3 min, and the PBS was aspirated. Two additional times, the cells in each tube were washed with ice-cold PBS as follows: 1 mL of ice-cold PBS was added, the tube was vortexed gently then centrifuged at 500g for 3 min, and the PBS was aspirated. Finally, 1 mL of ITSI Lysis Buffer (containing inhibitors) was added to each sample. Each tube was vortexed for 10 seconds, incubated on ice for 5 min, vortexed for 5 seconds, incubated on ice for 5 min, vortexed for 5 seconds, and then immediately placed on dry ice. Samples were stored at -80 degrees Celsius, packed in dry ice, and shipped overnight to ITSI Biosciences.

Kinase enrichment for proteomics and phospho-proteomics screen

Ammonium bicarbonate was purchased from Fisher Scientific, Pittsburgh, PA, and ammonium acetate was purchased from Sigma, St. Louis, MO. Cells were lysed using ITSI kinase lysis buffer and kinases were enriched according to the manufacturer's protocol (ITSI Kinase Enrichment Kit, ITSI Biosciences, Johnstown, PA). Briefly, cells were vortexed to lyse the cells and centrifuged at 12,000 x g at 4°C for 10 min to clarify the solution. Supernatant was transferred to a new tube and buffer exchanged into labeling buffer using a centrifugal desalting column. After the buffer exchange, phosphatase inhibitor cocktail was added. Protein assay was performed using the ITSI ToPA protein assay reagent (ITSI Biosciences, Johnstown, PA). The samples were diluted in labeling buffer to 2mg/ml. Then, 1mg was transferred to a new tube for labeling, and magnesium chloride was added at a concentration of 20mM. To enrich for kinases, desthiobiotin-ATP or -ADP were added to each sample and then each sample was incubated at room temperature for 10 minutes. After labeling, the samples were diluted 50:50 with 10M urea in lysis buffer. The samples were reduced with TCEP (Fisher Scientific, Pittsburgh, PA) and alkylated with iodoacetamide (GE Healthcare Biosciences, Piscataway, NJ). A buffer exchange was performed using a desalting column equilibrated with digestion buffer (1M urea in 20mM Tris buffer, pH 8.0). To each tube, 50ul of high capacity streptavidin resin was added. Then, the tubes were mixed for 2 hours at room temperature to capture the labeled proteins. The beads were washed 3 times with 500 µl of lysis buffer, 4 times with 500 µl of TBS, and 4 times with HPLC

grade water. After the last wash, the beads were suspended in 50ul of iTRAQ dissolution buffer with 5ug of sequencing grade trypsin. The tubes were incubated overnight at 37°C. After digestion, the peptides were transferred to a new tube and additional extraction was performed with 75ul of extraction buffer. The solution was dried. Then iTRAQ labeling was immediately commenced.

iTRAQ labeling and mass spectrometry analysis for proteomics and phospho-proteomics screen

Equal µg of control and treated samples were labeled with six iTRAQ™ labels (113 - 118) for 2 hours at room temperature. Labeled peptides were mixed and fractionated using SCX column to reduce the sample complexity and desalt the iTRAQ reagents. SCX separated peptides were dried in a speedvac and reconstituted in 5% Acetonitrile (Fisher Scientific, Pittsburgh, PA) / 0.1% Formic acid (Sigma, St. Louis, MO) and loaded onto a PicoFrit C18 nanospray column (New Objective, Woburn, MA) using a Thermo Scientific Surveyor Autosampler operated in no waste injection mode. Peptides were eluted from the column using a linear Acetonitrile gradient from 2% to 40% over 260 minutes into a LTQ XL mass spectrometer (Thermo Scientific) via a nanospray source with the spray voltage set to 1.8kV and the ion transfer capillary set at 180ºC. A data-dependent Top 3 method was used where a full MS scan from m/z 400-1500 was followed by MS/MS scans on the three most abundant ions. Each ion was subjected to CID for peptide identification followed by PQD for iTRAQ quantitation.

Database search and Kinase Enrichment Analysis (KEA) for proteomics and phospho-proteomics screen

Protein identification and phosphopeptide localization were determined with the Proteome Discoverer 1.3 software as previously described (El-Bayoumy, Das et al. 2012). Briefly, the raw data files were searched utilizing SEQUEST algorithm in Proteome Discoverer 1.3 (Thermo Scientific) against the most recent species-specific FASTA database for human downloaded from NCBI. Trypsin was the selected enzyme allowing for up to two missed cleavages per peptide; Methylthio Cysteine, N-terminal iTRAQ 8-plex, and Lysine iTRAQ 8-plex were used as static modifications and oxidation of Methionine, phosphorylation on S, Y, and T as a variable modification. Proteins were identified when two or more unique peptides had X-correlation scores greater than 1.5, 2.0, and 2.5 for respective charge states of +1, +2, and +3. Kinase Enrichment Analysis (KEA) was computationally performed using previously described software and protocol (Lachmann and Ma'ayan 2009). Only proteins that were identified with a minimum of five peptides were included in KEA.

Analysis of results from mass spectrometry-based proteomics and phospho-proteomics screen

Candidates were identified as proteins and phospho-peptides with greater than two-fold change between uncorrected and gene-corrected primary FANCA patient fibroblasts. Each candidate was thoroughly researched using UniProt and Gene (NCBI) databases, followed by PubMed search. Previously discovered

roles in cellular processes were thoroughly annotated for each candidate, with a particular focus on mitosis, DNA repair, apoptosis, and cancer.

Immunoblotting to validate candidates of mass spectrometry-based screen

Whole-cell extracts of uncorrected and gene-corrected primary FANCA patient fibroblasts were prepared using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific), followed by denaturation in SDS sample buffer. Proteins were resolved by SDS-PAGE, transferred to methanol-activated PVDF membranes, and immunoblotting was performed with the indicated antibodies. Membranes were developed via ECL reaction. The antibodies used include anti-BRCA1 (Abcam), anti-SKI (Santa Cruz), and anti-CoxIV (Cell Signaling).

Live imaging of taxol-challenged primary FANCA patient fibroblasts

Uncorrected and gene-corrected primary FANCA patient fibroblasts generated as previously described were plated at approximately 20,000 cells per well in a Hi-Q4 imaging dish (Ibidi, Munich, Germany). After two days, time-lapse phase-contrast imaging of cells was performed using a BioStation IM-Q time-lapse imaging system (Nikon, Melville, NY) equipped with a 20x 0.8 NA Plan Fluor objective lens. To visualize the outcome of SAC arrest in FANCA-deficient cells, the cell culture media was aspirated and replaced with media containing 200 nM taxol immediately prior to placing the imaging dish on the microscope. Time-lapse live-cell microscopy was performed for 48 hours with images captured every two minutes, and a total of 5 z-sections were captured at 2 um spacing.

Data acquisition was performed using BioStation IM software (Version:2.10 Build:131 or Version:2.21 Build:144), and manual quantification of dividing cells was performed utilizing NIS-Elements AR Analysis 4.10.02 or NIS-Elements Viewer 4.20 microscope imaging software (Nikon). The outcome of SAC arrest in response to prolonged exposure to taxol was recorded. The outcome was either apoptosis (which was observed as the formation of numerous round bodies coating the cell, followed by immediate progression to cell death) or SAC failure (which was observed as the initiation of cytokinesis, followed by the generation of one or two interphase multinucleated cells). Additionally, timepoints were recorded for the onset of mitosis (defined by the initiation of nuclear envelope breakdown and nuclear remodeling) and the end of the SAC (defined by the initiation of either apoptosis or cytokinesis). Based on these timepoints, the duration of SAC arrest was quantified.

Live imaging of untreated primary FANCA patient fibroblasts

Uncorrected and gene-corrected primary FANCA patient fibroblasts generated as previously described were plated at approximately 20,000 cells per well in a Hi-Q4 imaging dish (Ibidi, Munich, Germany). After two days, time-lapse phase-contrast imaging of cells was performed using a BioStation IM-Q time-lapse imaging system (Nikon, Melville, NY) equipped with a 20x 0.8 NA Plan Fluor objective lens. Prior to experiments visualizing unperturbed mitosis, the media was replaced with fresh media prior to placing the imaging dish on the microscope. Time-lapse live-cell microscopy was performed for 24 hours with

images captured every two minutes, and a total of 5 z-sections were captured at 2 um spacing. Data acquisition was performed using BioStation IM software (Version:2.10 Build:131 or Version:2.21 Build:144), and manual quantification of dividing cells was performed utilizing NIS-Elements AR Analysis 4.10.02 or NIS-Elements Viewer 4.20 microscope imaging software (Nikon). For the untreated cells, timepoints were recorded for numerous stages of mitosis and cytokinesis including the onsets of prophase, prometaphase, metaphase, anaphase, and cleavage furrow formation, as well as the completion of telophase. Additionally, the presence or absence of numerous phenotypic defects in unperturbed mitosis and cytokinesis were noted. Prophase onset was defined by the initiation of nuclear envelope (NE) breakdown in conjunction with remodeling of nucleoli. The onset of prometaphase was recorded as the timepoint at which the NE had completely dissolved and chromatin had completely condensed. Metaphase was defined by the formation of the metaphase plate. The initiation of anaphase was defined by the onset of chromosome segregation. The onset of cytokinesis was defined by the first appearance of the cleavage furrow. The completion of telophase was defined as the timepoint in each daughter cell at which NE re-assembly had concluded. Phenotypic defects recorded for each cell included absence of a clear metaphase plate, rotation of the metaphase spindle (visualized based on the morphology and positioning of the metaphase plate), rotation of the anaphase spindle (visualized as a vertical offset between newly forming daughter cells as the dividing cell entered anaphase), asynchronous telophase NE re-assembly, and abnormal vesicle formation during cytokinesis.

Statistics

Statistical analysis was performed using GraphPad Prism and Microsoft Excel software, and results with *P* values less than 0.05 were considered significant. For siRNA experiments, results for each siRNA were compared with negative control siRNA by one-way ANOVA followed by post-hoc Fisher's LSD test or Bonferroni's multiple comparison test. For taxol challenge of primary FA patient fibroblasts (imaging and flow cytometry) and pericentrin immunostaining in siRNA-transfected HeLa cells and FA patient fibroblasts, one-way ANOVA was followed by post-hoc Bonferroni's multiple comparison test to compare each sample with the appropriate negative control. In these experiments utilizing primary FA patient fibroblasts, FA fibroblast lines were compared with the average of equal numbers of replicates of two healthy control primary fibroblast lines. Two-tailed t-tests were used for all experiments comparing a single experimental sample with a negative control. For live-cell video microscopy experiments, a two-tailed t-test was applied to each comparison. Additionally, for the phenotypic defects observed in untreated FANCA patient fibroblasts, correlation statistics were performed to calculate Pearson *r* values and corresponding *P* values. Finally, in hypersensitivity assays, the effects of FANCA gene status and taxol concentration were analyzed using two-way ANOVA followed by post-hoc Tukey's multiple comparison test.

CHAPTER THREE

THE FA SIGNALING NETWORK IS ESSENTIAL FOR THE MITOTIC SAC

Introduction

The FA signaling network is essential for the maintenance of genomic integrity, with aneuploidy frequently developing in the absence of an intact FA pathway. FA patients are predisposed to the hematologic neoplasms MDS and AML, and studies examining the cytogenetic profiles of bone marrow (BM) aspirates from pre-leukemic and leukemic FA patients have noted that the development of complex, random aneuploidy generally precedes malignant transformation (Berger, Le Coniat et al. 1993, Alter, Caruso et al. 2000, Cioc, Wagner et al. 2010, Mehta, Harris et al. 2010, Rochowski, Olson et al. 2012).

Our laboratory previously generated a *Fancc-/-; Fancg-/-* murine model in order to study the functional interaction of the Fancc and Fancg genes in the pathogenesis of FA. This was the first FA murine model to spontaneously develop bone marrow failure and the hematopoietic malignancies which are characteristic of FA. As in patients with FA, complex chromosomal abnormalities were observed when spectral karyotyping was performed on BM aspirates from *Fancc-/-; Fancg-/-* mice, and myelodysplasia was observed upon histopathologic examination post-mortem. Thus, our laboratory's observations in the *Fancc-/-; Fancg-/-* murine model confirm that the presence of complex random aneuploidy is correlated with the development of myeloid malignancies in FA (Pulliam-Leath, Ciccone et al. 2010).

An additional study performed in the *Fancc-/-; Fancg-/-* murine model suggests a connection with the mitotic SAC. In a gene chip-based screen, the entire transcriptome was quantified in bone marrow cells taken from WT, *Fancc-/-*, *Fancg-/-*, and *Fancc-/-; Fancg-/-* mice. The dysregulated transcripts encoded a number of known mitotic SAC regulators, notably the MCC proteins Mad2 and BubR1 (A. Pulliam-Leath, S. Ciccone, G. Nalepa, G. Bagby, D. W. Clapp, unpublished data).

This study led us to hypothesize that the human FA proteins are essential for the activity of the mitotic SAC and that the aneuploidy and oncogenesis observed in FA patients are at least partially caused by defective regulation of the mitotic SAC in FA-deficient cells. Since the mitotic SAC is an important tumor suppressor pathway that protects genomic integrity by ensuring accurate chromosome segregation during mitosis, we reasoned that a weakened SAC could provide a mechanistic explanation for the development of aneuploidy in FA-deficient cells. To investigate the role of the FA signaling network in the regulation of the mitotic SAC and the prevention of aneuploidy, we performed genome-wide RNAi-based experiments and numerous studies utilizing primary cells from patients with FA.

Results

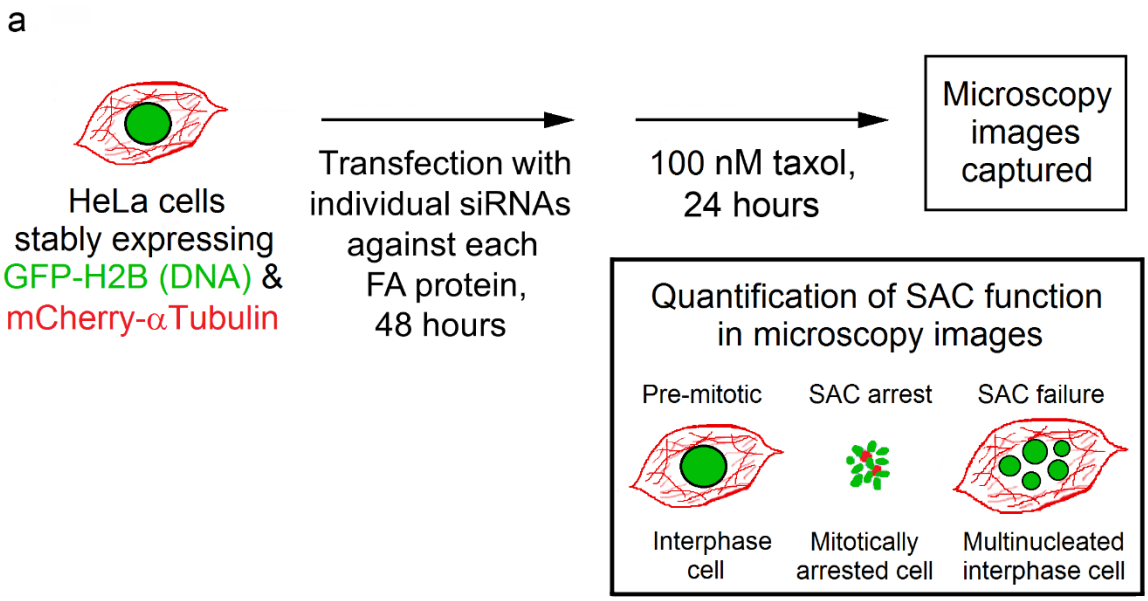
Genome-wide RNAi screen of known FA proteins for essential role in the activity of the mitotic SAC

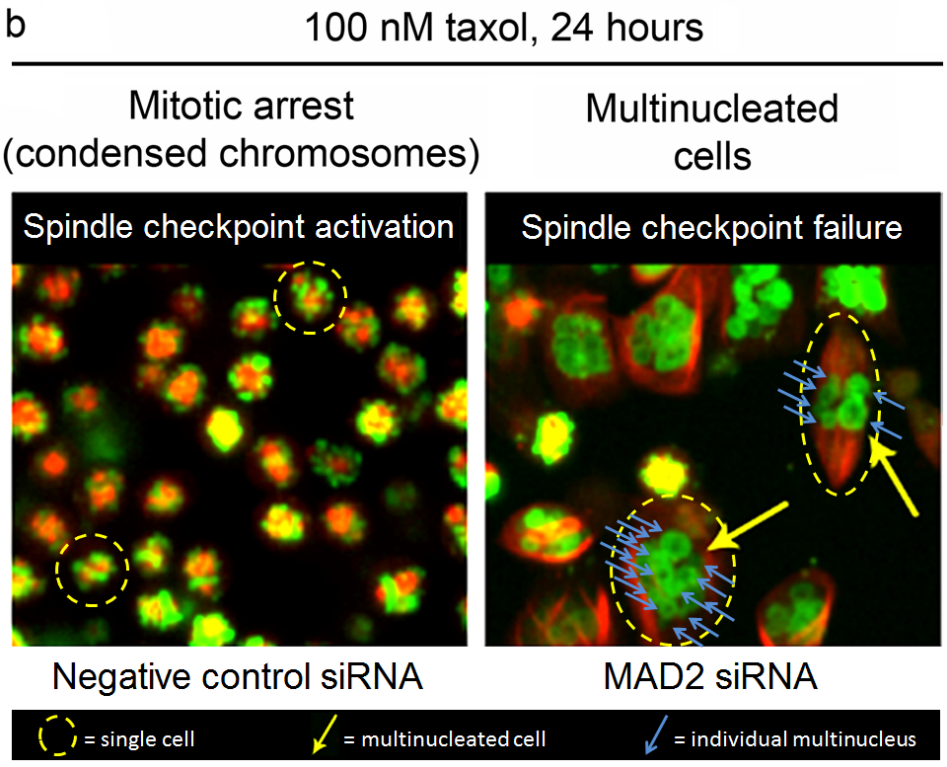
To assess the potential role of the FA signaling network in the activity of the mitotic SAC, we performed a functional RNAi screen which examined the status of the SAC upon knockdown of individual FA gene products (Figure 3-1). An siRNA library was generated which included siRNAs targeting all known human FA gene products. HeLa cells were reverse transfected with library siRNAs arrayed in a 96-well plate, challenged with taxol, fixed, and imaged using a BD Pathway automated microscope (Figure 3-1a). Then, the captured microscopy images were manually quantified using ImageJ.

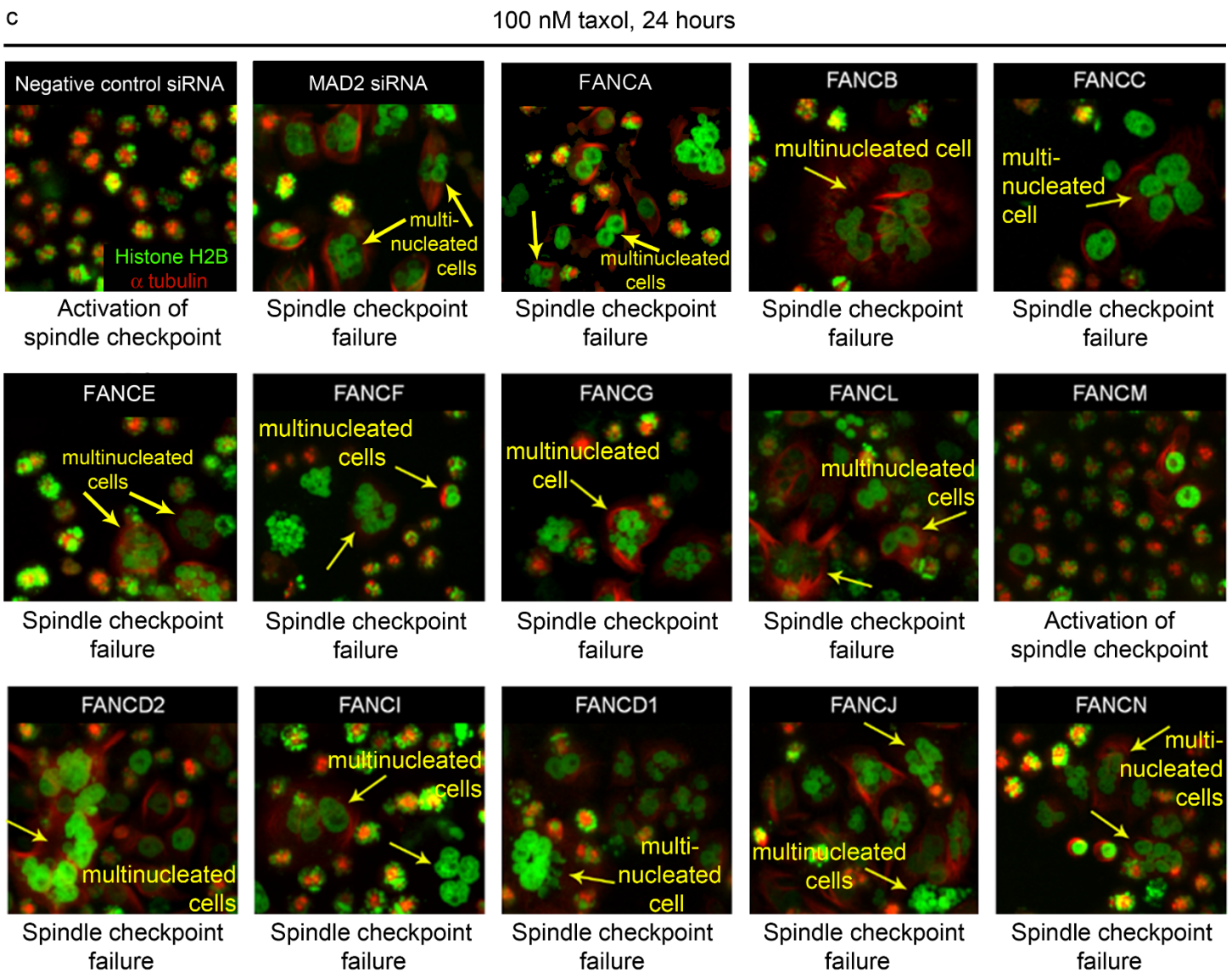
The chemotherapeutic agent taxol was used because it activates the mitotic SAC. As a microtubule stabilizer, taxol prevents remodeling of the mitotic spindle and results in incomplete formation of kinetochore-spindle fiber attachments. Due to the presence of unattached kinetochores, the SAC is activated in the presence of taxol and other drugs targeting the mitotic spindle. Negative control cells with a functional mitotic SAC were generated by transfection with a scrambled negative control siRNA, and positive control cells with an inactivated mitotic SAC were generated by transfection with an siRNA against the MCC protein MAD2. In response to taxol challenge, negative control cells arrested in prometaphase due to appropriate SAC activation (Figure 3-1b). In contrast, positive control cells generated by RNAi knockdown of MAD2 failed

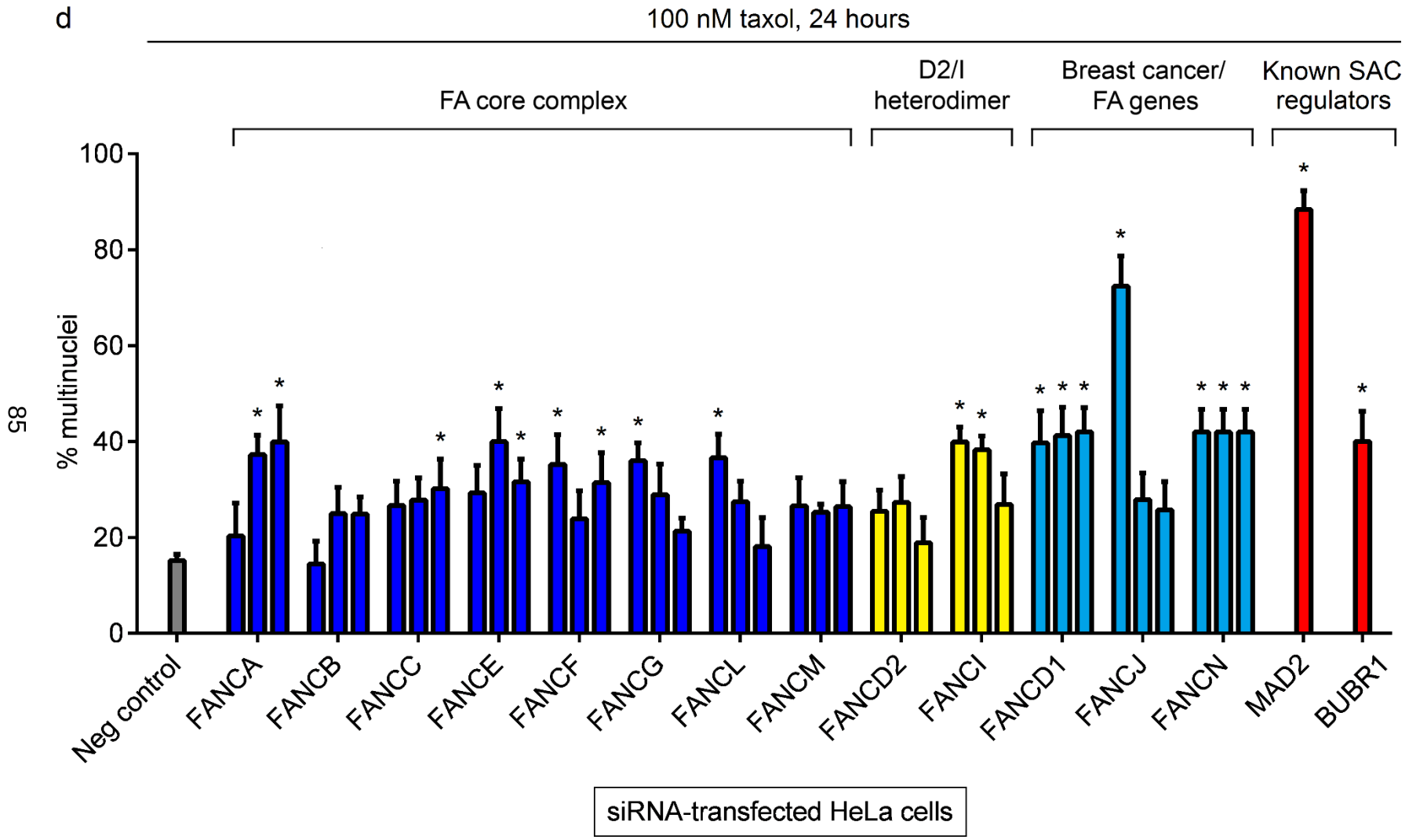
to maintain SAC arrest in taxol and became interphase multinucleated cells (Figure 3-1b).

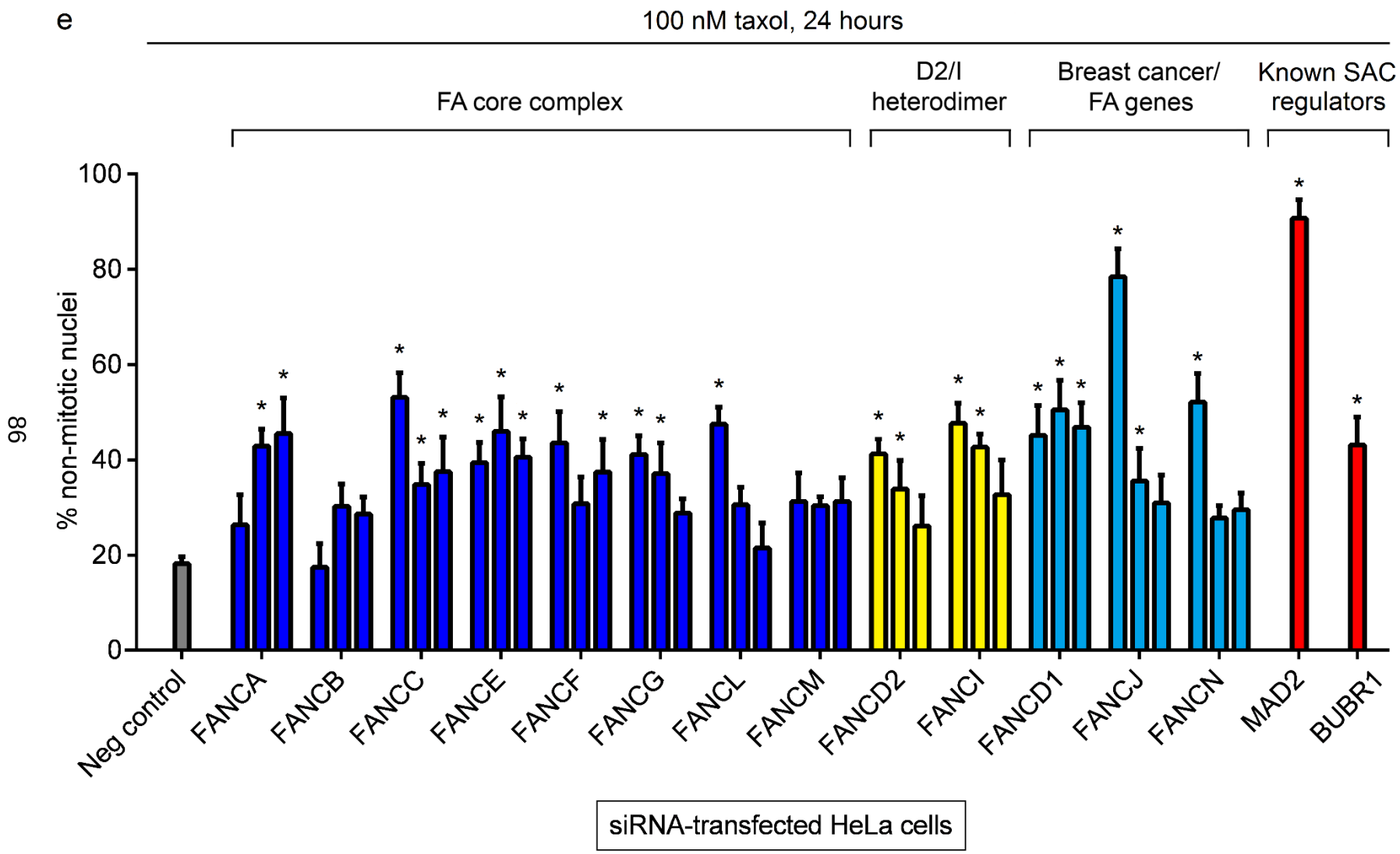
The screen was performed twice with two independent siRNA libraries. In the initial screen, a custom Silencer siRNA library was designed and three unvalidated siRNA sequences per genotype were tested against each of the thirteen known FA gene products. (The results of the initial screen are shown in Figure 3-1d, e.) In 2010 and 2011 respectively, FANCO and FANCP were identified as novel FA complementation groups (Vaz, Hanenberg et al. 2010, Kim, Lach et al. 2011). Since two new FA genes had been discovered and since Silencer siRNAs produced inconsistent results in our screen most likely due to inconsistent knockdown of targeted gene products, a second siRNA library was designed utilizing next-generation Silencer Select siRNAs. In the second screen, one validated or three unvalidated Silencer Select siRNA sequences were tested against each of the fifteen known FA gene products. (The results of the second screen are shown in Figure 3-1f, g.) Remarkably, we found that RNAi silencing of fourteen of the fifteen known FA genes inactivated the SAC, as evidenced by the generation of interphase multinucleated cells in response to taxol, similar to RNAi silencing of *MAD2* (Figure 3-1c, d, and e).

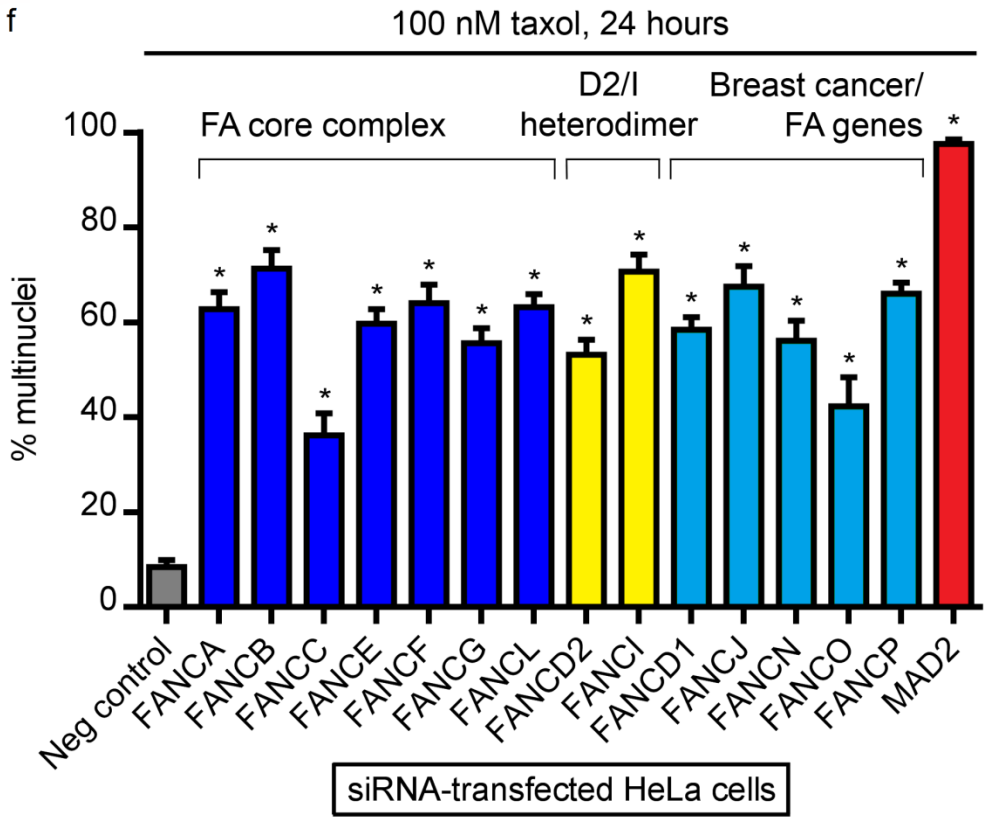

a
HeLa cells stably expressing GFP-H2B (DNA) & mCherry-αTubulin
Transfection with individual siRNAs against each FA protein, 48 hours
100 nM taxol, 24 hours
Microscopy images captured
Quantification of SAC function in microscopy images
Pre-mitotic
SAC arrest
SAC failure
Interphase cell
Mitotically arrested cell
Multinucleated interphase cell


b
100 nM taxol, 24 hours
Mitotic arrest (condensed chromosomes)
Multinucleated cells
Spindle checkpoint activation
Spindle checkpoint failure
Negative control siRNA
MAD2 siRNA
= single cell
= multinucleated cell
= individual multinucleus


c
100 nM taxol, 24 hours
Negative control siRNA
Histone H2B
α tubulin
Activation of spindle checkpoint
MAD2 siRNA
multi-nucleated cells
Spindle checkpoint failure
FANCA
multinucleated cells
Spindle checkpoint failure
FANCB
multinucleated cell
Spindle checkpoint failure
FANCC
multi-nucleated cell
Spindle checkpoint failure
FANCE
multinucleated cells
Spindle checkpoint failure
FANCF
multinucleated cells
Spindle checkpoint failure
FANCG
multinucleated cell
Spindle checkpoint failure
FANCL
multinucleated cells
Spindle checkpoint failure
FANCM
Activation of spindle checkpoint
FANCD2
multinucleated cells
Spindle checkpoint failure
FANCI
multinucleated cells
Spindle checkpoint failure
FANCD1
multi-nucleated cell
Spindle checkpoint failure
FANCJ
multinucleated cells
Spindle checkpoint failure
FANCN
multi-nucleated cells
Spindle checkpoint failure


d
100 nM taxol, 24 hours
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
Known SAC regulators
% multinuclei
100
80
60
40
20
0
Neg control
FANCA
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCM
FANCD2
FANCI
FANCD1
FANCJ
FANCN
MAD2
BUBR1
siRNA-transfected HeLa cells


e
100 nM taxol, 24 hours
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
Known SAC regulators
% non-mitotic nuclei
100
80
60
40
20
0
Neg control
FANCA
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCM
FANCD2
FANCI
FANCD1
FANCJ
FANCN
MAD2
BUBR1
siRNA-transfected HeLa cells


f
100 nM taxol, 24 hours
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
% multinuclei
100
80
60
40
20
0
Neg control
FANCA
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1
FANCJ
FANCN
FANCO
FANCP
MAD2
siRNA-transfected HeLa cells

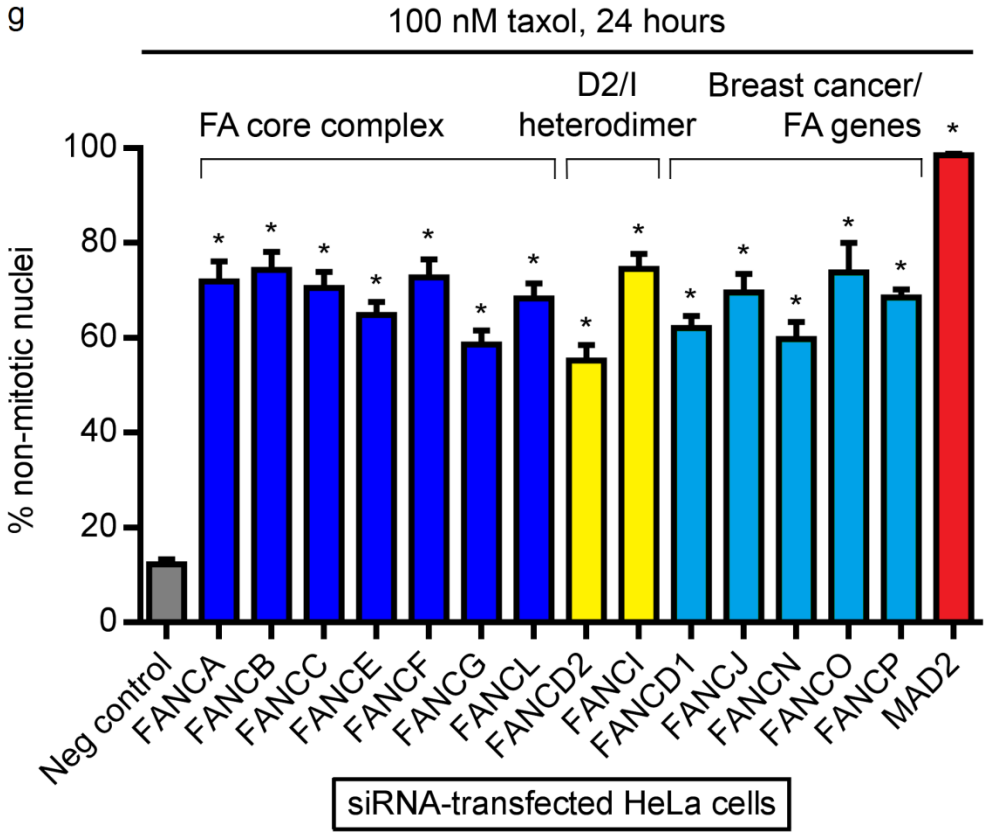

g
100 nM taxol, 24 hours
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
% non-mitotic nuclei
100
80
60
40
20
0
Neg control
FANCA
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1
FANCJ
FANCN
FANCO
FANCP
MAD2
siRNA-transfected HeLa cells

Figure 3-1. Genome-wide RNAi screen demonstrates that the FA signaling network is essential for the mitotic SAC. a) Schematic of the RNAi screen. The HeLa$^{GFP\text{-}H2B/mCherry\text{-}\alpha tubulin}$ cell line stably expresses GFP-tagged Histone H2B and mCherry-tagged α-tubulin, which label DNA and microtubules respectively. The three types of cell which were observed following challenge with 100 nM taxol were interphase, mitotic, and multinucleated cells. Interphase cells represent cells which have not entered mitosis, mitotic cells represent cells arrested at the mitotic SAC, and multinucleated cells represent cells which have failed to maintain arrest at the mitotic SAC. **b)** Examples of microscopy-based phenotypes. Negative control siRNA-transfected HeLa$^{GFP\text{-}H2B/mCherry\text{-}\alpha tubulin}$ cells arrest in prometaphase upon taxol exposure, indicating an active mitotic SAC. Positive control MAD2 siRNA-transfected cells become multinucleated interphase cells in response to taxol challenge, indicating SAC failure in each multinucleated cell and weakened mitotic SAC activity in the total population of cells. Each dotted circle encloses a single cell. Larger yellow arrows indicate multinucleated cells and smaller blue arrows indicate individual multinuclei. Original magnification is ×200 (BD Pathway 855). **c)** Representative images of cells transfected with indicated siRNAs targeting individual FA gene products and subsequently challenged with taxol. Weakened SAC activity evidenced by an increased rate of multinucleation following taxol challenge was observed for HeLa cells transfected with siRNAs against MAD2 and FA gene products. Original magnification is ×200 (BD Pathway 855). **d and e)** Quantification of microscopy-based results from the initial siRNA screen. For ten of thirteen FA gene products tested, FA-knockdown HeLa cells exhibit weakened SAC activity as evidenced by an increase in the % multinuclei and % non-mitotic nuclei in response to taxol, compared with negative control. An asterisk denotes $P < 0.05$ (1-way ANOVA with post-hoc Fisher's LSD test), all bars represent mean values ± SEM, and $n = 5$ independent transfections per siRNA. **f and g)** Quantification of microscopy-based results. For fourteen of fifteen FA gene products tested, FA-knockdown HeLa cells exhibit weakened SAC activity as evidenced by an increase in the % multinuclei and % non-mitotic nuclei in response to taxol, compared with negative control. For each FA gene product, only the single siRNA producing the strongest positive result is graphed in panels **f and g**. An asterisk denotes $P < 0.0001$ (1-way ANOVA with post-hoc Bonferroni's correction), all bars represent mean values ± SEM, 3 independent transfections were performed, and $n = 9$ microscopic fields per siRNA.

Verification of protein target knockdown by siRNAs

To verify that the siRNAs used in the RNAi-based screen successfully achieved knockdown of their respective FA protein targets, HeLa cells were transfected with the indicated siRNA or negative control siRNA for 48 hours. Then, the level of the indicated protein target was assessed by immunoblotting followed by densitometry. Effective knockdown was detected for the siRNA against MAD2 and for siRNAs against thirteen of the fourteen FA gene products which were positive hits in the RNAi-based screen, validating these siRNAs. The siRNAs against FANCF and FANCM could not be validated, as commercial antibodies which were tested failed to detect a band of the appropriate size.

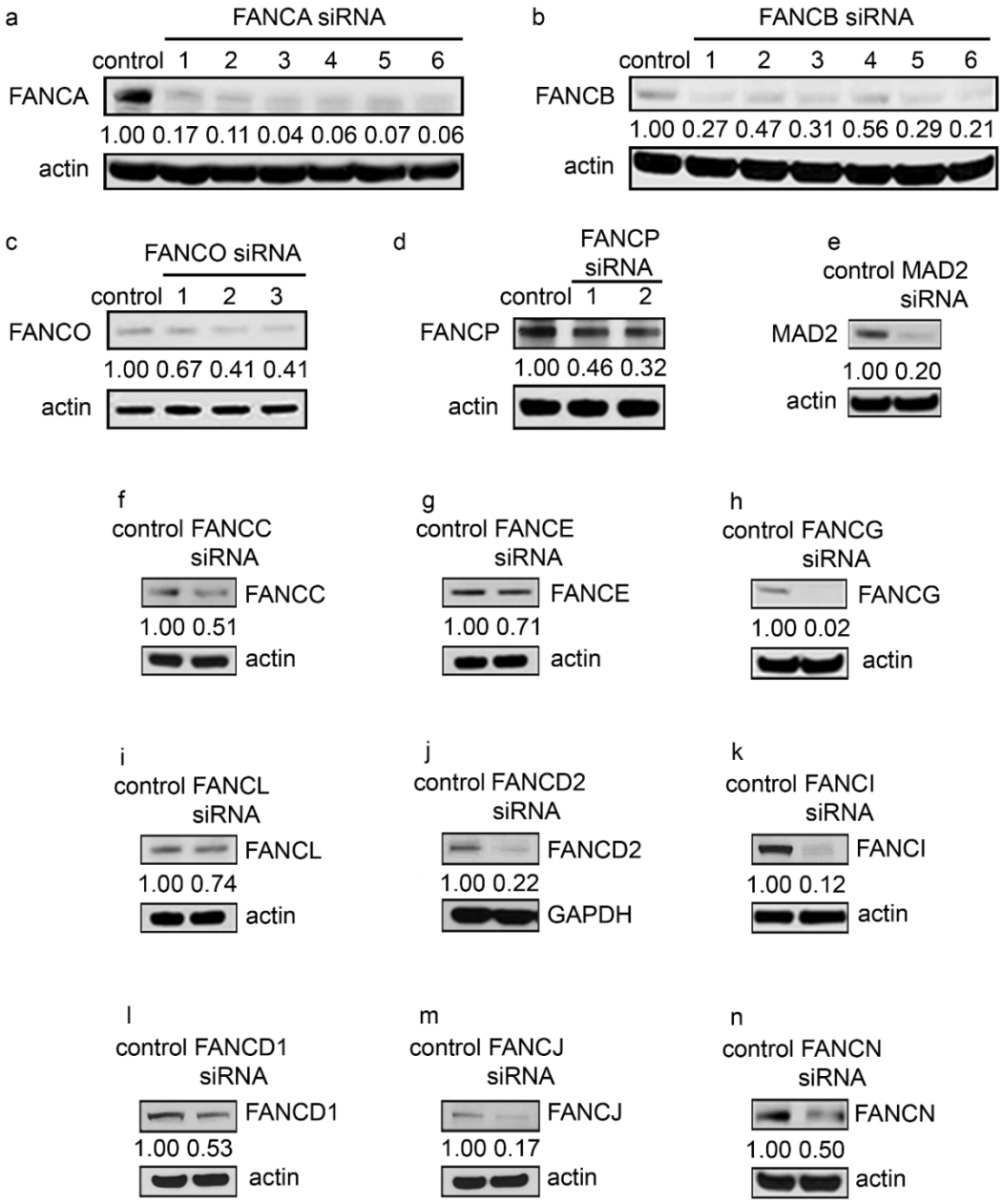


Figure 3-2. Immunoblotting experiments validate the siRNAs used in the mitotic SAC RNAi screen. a) Six unique siRNA sequences result in knockdown of the FANCA protein in FANCA-transfected HeLa cells compared with negative control-transfected HeLa cells. **b, c, and d)** Six unique siRNA sequences against FANCB, three unique siRNA sequences against FANCO, and two unique siRNA sequences against FANCP result in knockdown of the respective protein target. **e)** Individual siRNAs against MAD2 and nine additional FA proteins result in knockdown of the indicated respective protein target. For each immunoblot, numbers represent densitometry-based quantification of the target band intensities normalized to loading control. Band intensities are reported in arbitrary units with negative control siRNA assigned to a value of 1.

Demonstration that multiple siRNAs against the same FA protein target result in mitotic SAC failure

For four FA gene products, multiple unique siRNA sequences were tested, and more than one siRNA against the same FA gene product resulted in the phenotype of mitotic SAC failure. Additionally, when multiple siRNAs were tested, the percentage of cells exhibiting SAC failure correlated with the degree of protein target knockdown detected by immunoblotting (compare Figure 3-3 below with Figure 3-2 above). These data are important for two reasons. First, the use of multiple unique siRNA sequences targeting the same FA gene product at different locations affirms that the weakened SAC phenotype results from knockdown of the specific FA gene product targeted by the siRNA rather than from an off-target effect. Second, the use of siRNAs producing variable levels of FA protein target knockdown enables the following observations: a) the degree of SAC phenotype appears to correlate with the degree of FA protein target knockdown and b) a certain threshold of knockdown may be required before a SAC phenotype is observed.

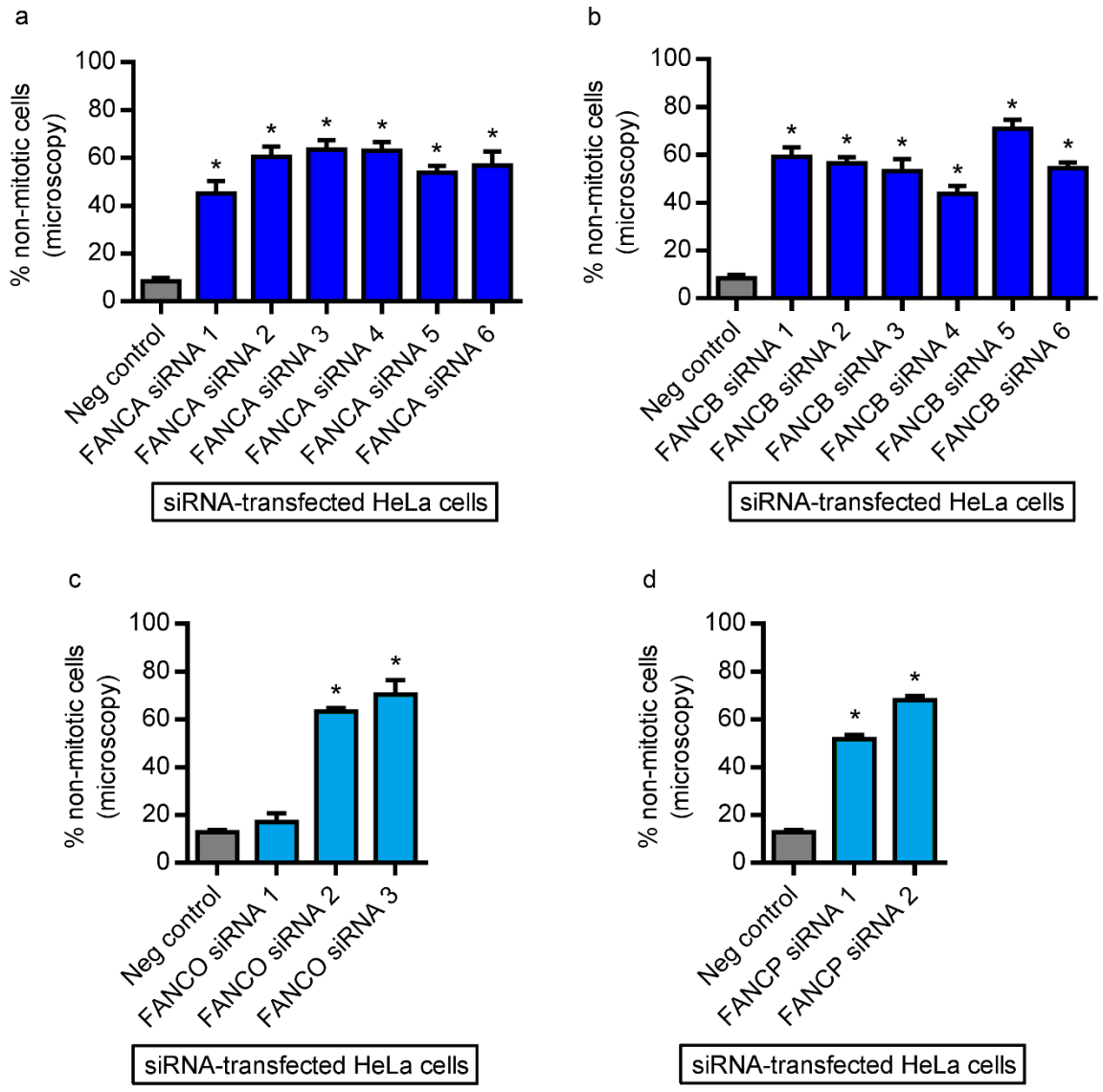
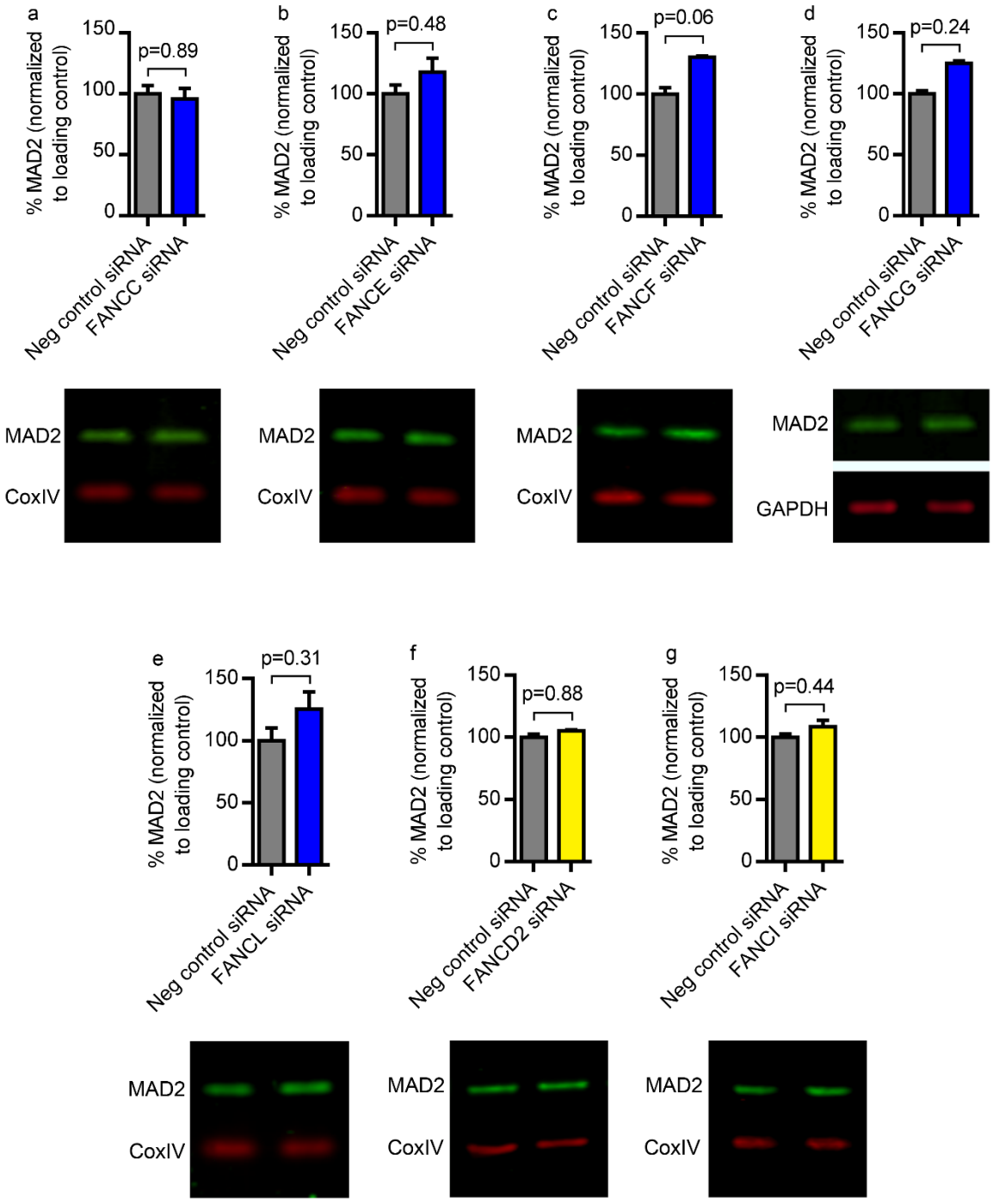


Figure 3-3. Microscopy-based quantification of SAC failure resulting from knockdown of the same FA protein target by multiple siRNAs. a) Six of six unique siRNA sequences against FANCA, **b)** six of six against FANCB, **c)** two of three against FANCO, and **d)** two of three against FANCP cause SAC failure in taxol-challenged HeLa$^{GFP\text{-}H2B/mCherry\text{-}\alpha tubulin}$ cells. An asterisk denotes $P < 0.0001$ (1-way ANOVA with post-hoc Bonferroni's correction), $n \geq 3$ microscopic fields per siRNA, and all bars represent mean values ± SEM.

Verification that siRNAs do not have an off-target effect on MAD2

Off-target siRNA effects are a risk in RNAi-based screens. In previous RNAi-based screens of mitotic SAC activity, MAD2 has been shown to be particularly susceptible to nonspecific knockdown by siRNAs designed to target other gene products (Sigoillot, Lyman et al. 2012). Thus, we wanted to verify that the siRNAs used in our RNAi-based screen did not exert a nonspecific effect on the level of MAD2. For several FA gene products, multiple unique siRNA sequences targeting the same FA gene product resulted in the phenotype of mitotic SAC failure (see Figure 3-3 above), making it unlikely that an off-target effect is responsible for this phenotype. For the other ten FA gene products which were identified as SAC regulators in the screen, quantitative immunoblotting was performed to show that MAD2 is not nonspecifically knocked down by siRNAs targeting FA gene products. HeLa cells were transfected with either negative control siRNA or the indicated FA siRNA for 48 hours, cell lysates were prepared, and infrared-based quantitative immunoblotting was performed using a LI-COR Odyssey CLx imager.


a
% MAD2 (normalized to loading control)
150
100
50
0
p=0.89
Neg control siRNA
FANCC siRNA
b
% MAD2 (normalized to loading control)
150
100
50
0
p=0.48
Neg control siRNA
FANCE siRNA
c
% MAD2 (normalized to loading control)
150
100
50
0
p=0.06
Neg control siRNA
FANCF siRNA
d
% MAD2 (normalized to loading control)
150
100
50
0
p=0.24
Neg control siRNA
FANCG siRNA
MAD2
CoxIV
MAD2
CoxIV
MAD2
CoxIV
MAD2
GAPDH
e
% MAD2 (normalized to loading control)
150
100
50
0
p=0.31
Neg control siRNA
FANCL siRNA
f
% MAD2 (normalized to loading control)
150
100
50
0
p=0.88
Neg control siRNA
FANCD2 siRNA
g
% MAD2 (normalized to loading control)
150
100
50
0
p=0.44
Neg control siRNA
FANCI siRNA
MAD2
CoxIV
MAD2
CoxIV
MAD2
CoxIV

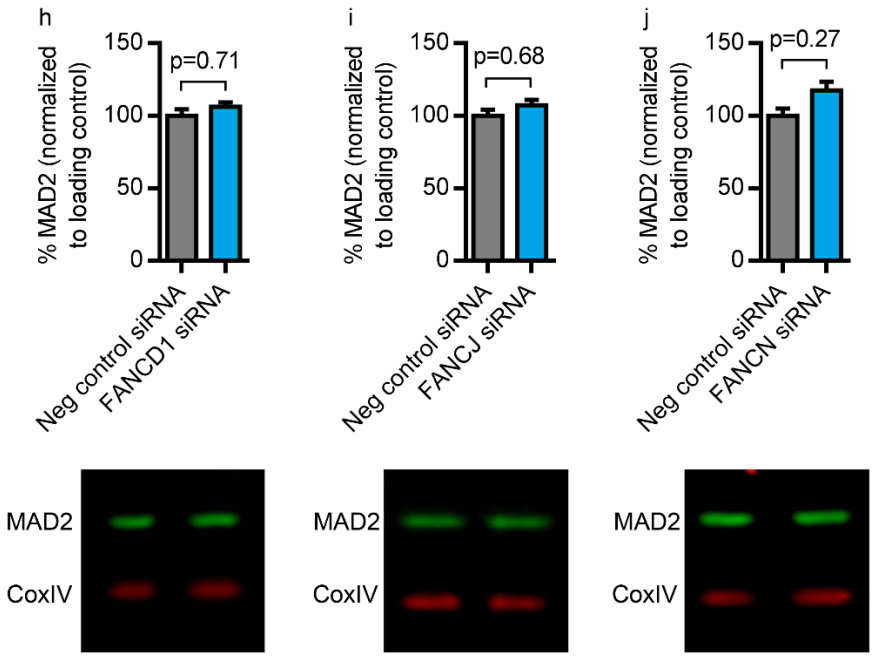
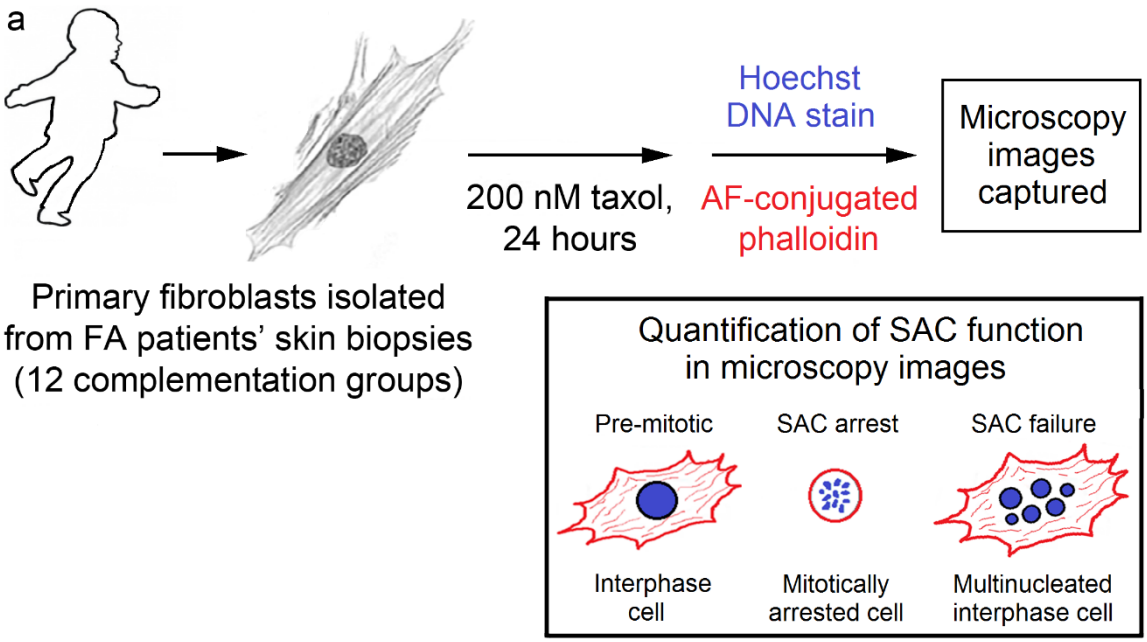


Figure 3-4. Quantitative immunoblotting verifies that FA siRNAs do not have an off-target effect on MAD2. The level of MAD2 is not significantly different between negative control siRNA-transfected and FA siRNA-transfected HeLa cells for the ten indicated siRNAs targeting different FA proteins ($P > 0.05$ by two-tailed t-test). *P* values are noted on each graph, $n = 3$ independent transfections and immunoblots per siRNA, and all bars represent mean values ± SEM. MAD2 target band intensities were quantified using LI-COR Image Studio software and normalized to loading control. Band intensities are reported in arbitrary units with the average MAD2 intensity in negative control siRNA-transfected samples assigned to a value of 100%.

Quantification of mitotic SAC failure in primary cells from FA patients

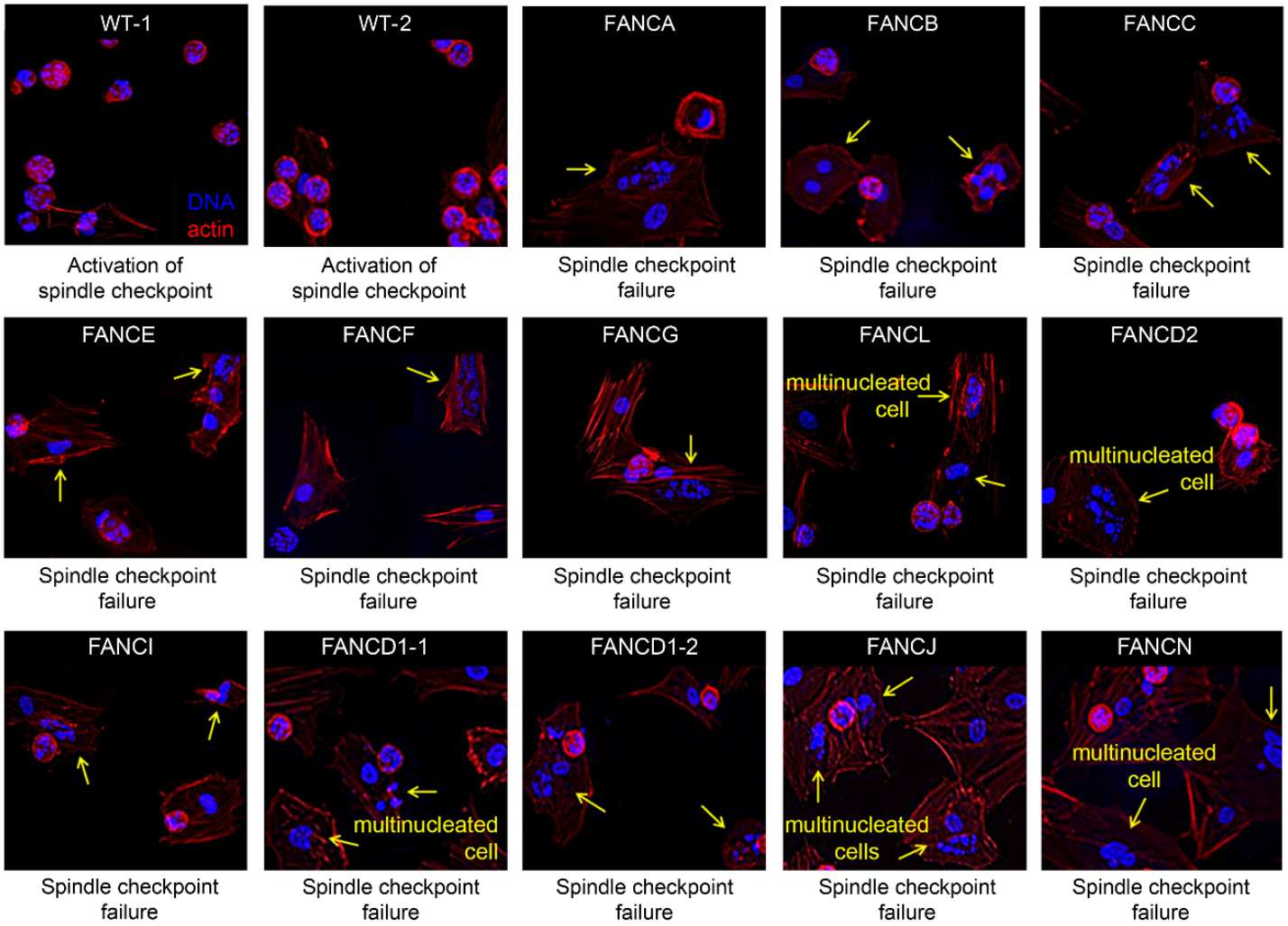
To confirm the results of the RNAi screen, we obtained primary fibroblasts from FA patients of twelve different FA complementation groups from our collaborator Helmut Hanenberg. These cells were challenged with taxol, fixed, stained in order to visualize DNA and actin, and imaged (Figure 3-5a). Microscopy images were manually quantified. Additionally, taxol-challenged primary fibroblasts from FA patients were stained with phospho-histone H3 (S10) and analyzed via flow cytometry. Phosphorylation of serine 10 of the nucleosome component histone H3 temporally corresponds with the initiation of chromosome condensation at the onset of mitosis, the concentration of phospho-histone H3 (S10) is highest in metaphase, and a global de-phosphorylation of histone H3 occurs at the completion of mitosis (Hans and Dimitrov 2001). Thus, phospho-histone H3 (S10) is an excellent marker for flow cytometry-based quantification of mitotic arrest. Consistent with the mitotic SAC RNAi screen results, SAC failure evidenced by increased multinucleation and increased non-mitotic cells was observed for taxol-challenged primary fibroblasts of all tested FA complementation groups, while fibroblasts from healthy individuals exhibited normal mitotic SAC arrest (Figure 3-5).

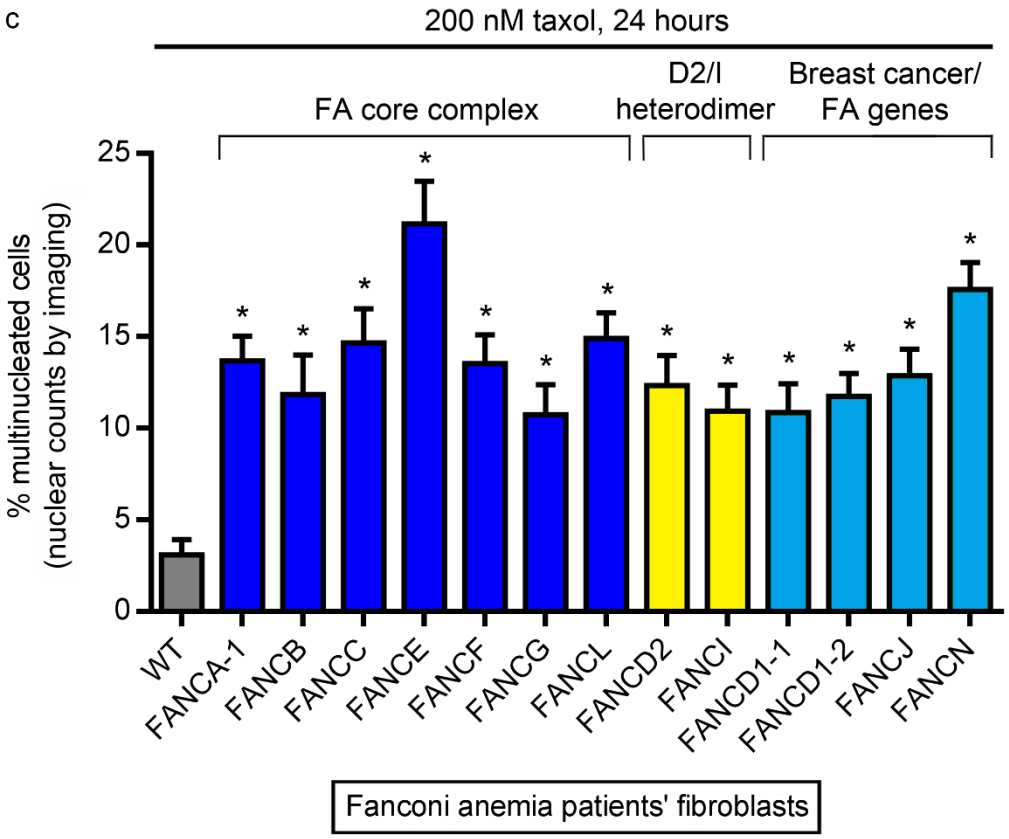

a
Hoechst
DNA stain
Microscopy
images
captured
200 nM taxol,
24 hours
AF-conjugated
phalloidin
Primary fibroblasts isolated
from FA patients' skin biopsies
(12 complementation groups)
Quantification of SAC function
in microscopy images
Pre-mitotic
SAC arrest
SAC failure
Interphase
cell
Mitotically
arrested cell
Multinucleated
interphase cell

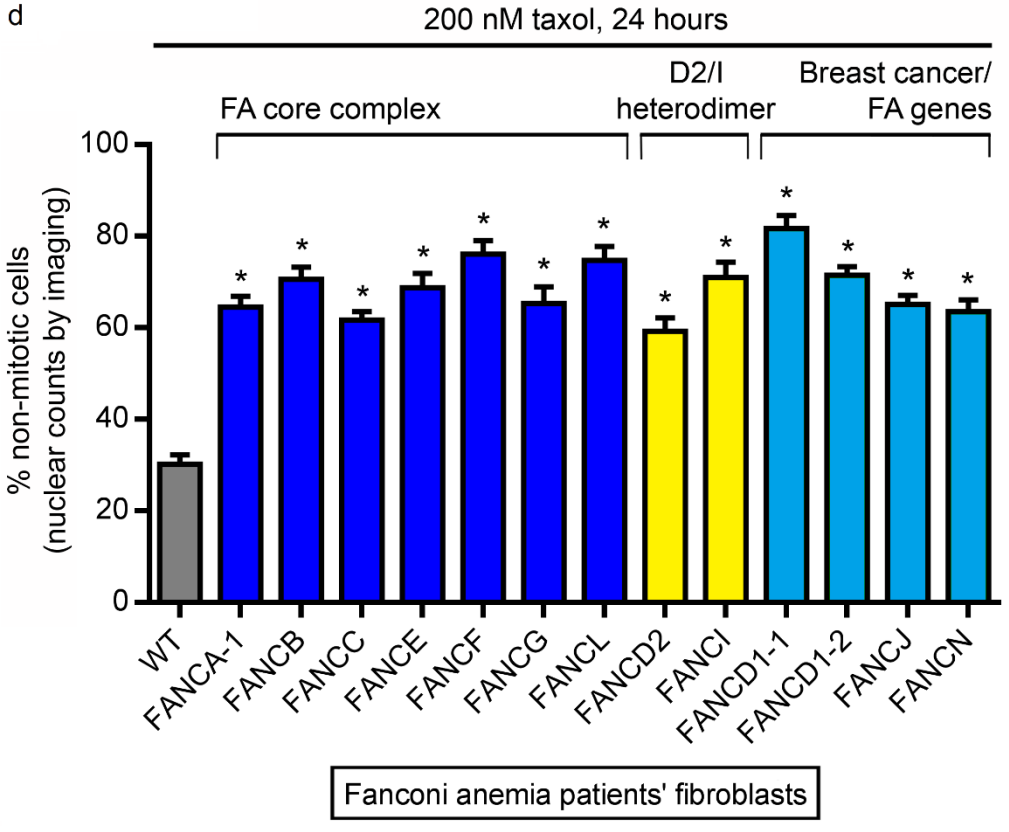
b

Fanconi anemia patients' fibroblasts

200 nM taxol, 24 hours




c
200 nM taxol, 24 hours
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
% multinucleated cells (nuclear counts by imaging)
25
20
15
10
5
0
WT
FANCA-1
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1-1
FANCD1-2
FANCJ
FANCN
Fanconi anemia patients' fibroblasts


d
200 nM taxol, 24 hours
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
% non-mitotic cells (nuclear counts by imaging)
100
80
60
40
20
0
WT
FANCA-1
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1-1
FANCD1-2
FANCJ
FANCN
Fanconi anemia patients' fibroblasts

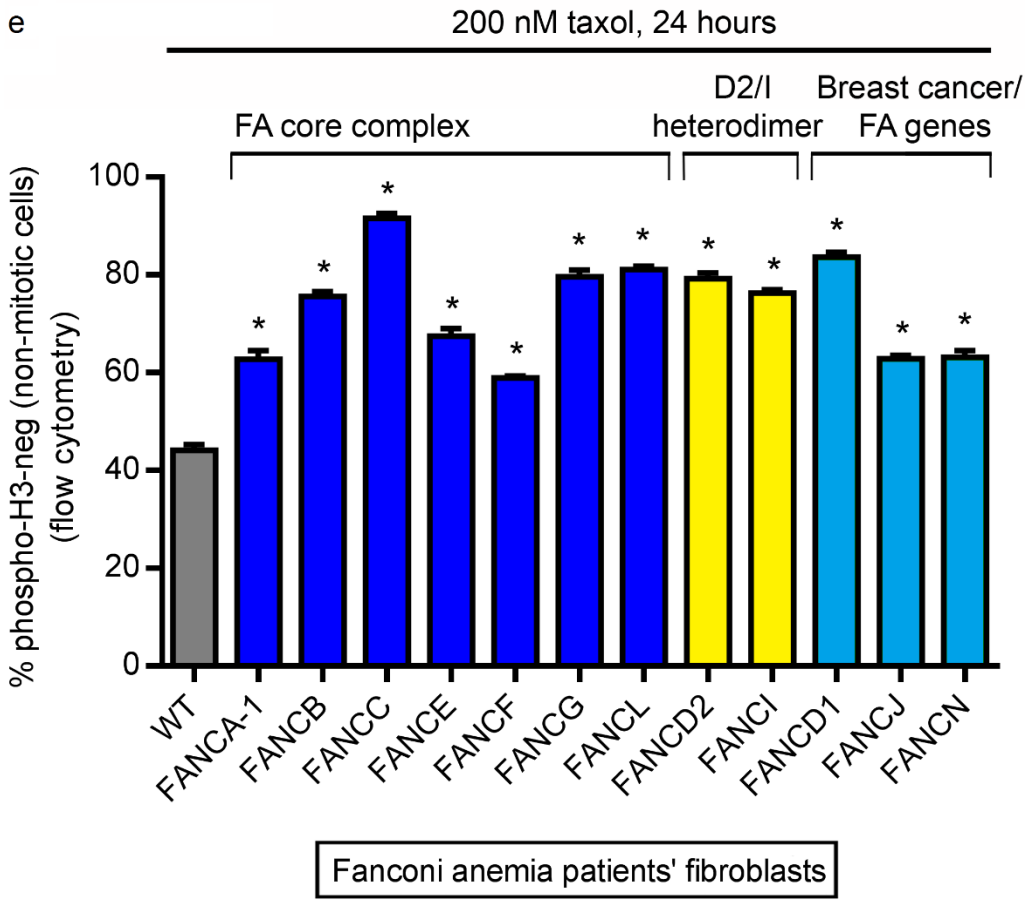
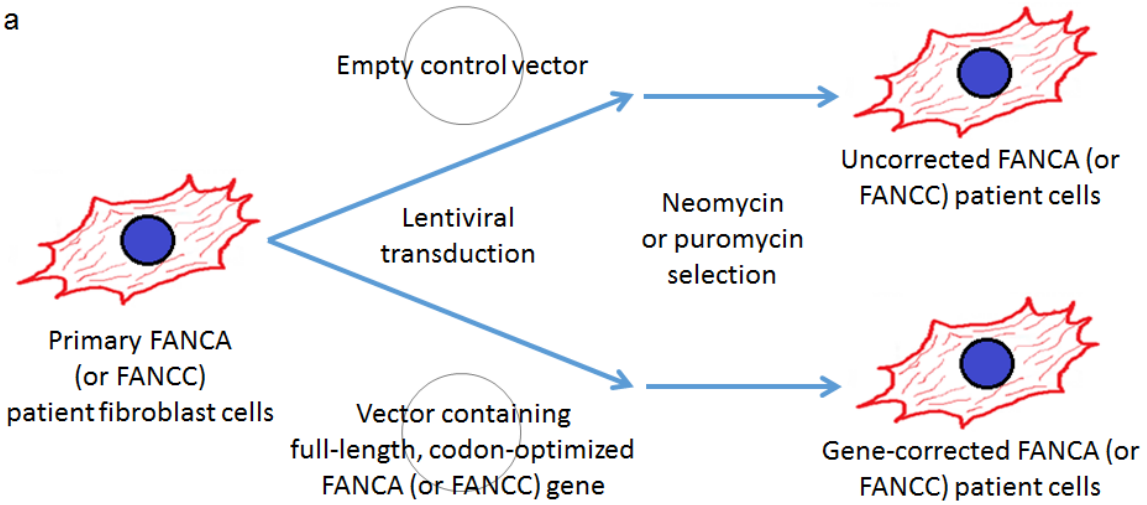
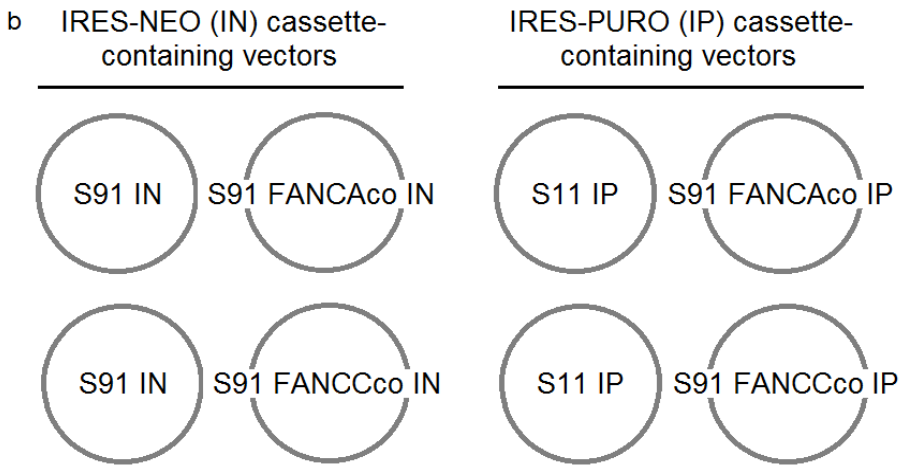

e
200 nM taxol, 24 hours
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
% phospho-H3-neg (non-mitotic cells) (flow cytometry)
100
80
60
40
20
0
WT
FANCA-1
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1
FANCJ
FANCN
Fanconi anemia patients' fibroblasts

Figure 3-5. Quantification of SAC failure in primary fibroblasts from FA patients confirms that the human FA signaling network is essential for the mitotic SAC. a) Schematic of microscopy-based mitotic SAC assessment in primary fibroblasts from FA patients. Hoechst 33342 and Alexa Fluor-conjugated phalloidin were used to label DNA and actin respectively. **b)** Representative images of taxol-challenged FA-deficient primary fibroblasts. FA fibroblasts fail to arrest in mitosis following taxol challenge and generate multinucleated cells (arrows), while WT (wild type) fibroblasts from healthy control patients demonstrate appropriate prometaphase arrest following taxol exposure. The two FANCD1 cell lines imaged in this panel (FANCD1-1 and FANCD1-2) are derived from two siblings carrying the same mutations in the *FANCD1/BRCA2* gene. A summary of the specific mutations detected in all of the primary FA patient fibroblasts used in this study appears in Table 2 in the Methods section. Original magnification of images is ×200 (Applied Precision personalDV). **c and d)** Quantification of microscopy-based results. In **c,** an asterisk denotes $P < 0.01$ (1-way ANOVA with post-hoc Bonferroni's correction); in **d,** an asterisk denotes $P < 0.0001$ (1-way ANOVA with post-hoc Bonferroni's correction); $n = 10–15$ microscopic fields per FA genotype, and all bars represent mean values ± SEM. **e**) Quantification of flow cytometry-based results. Taxol-challenged FA patient fibroblasts show decreased phospho-histone H3-positive mitotic cells and correspondingly increased phospho-histone H3-negative non-mitotic cells when analyzed by flow cytometry, confirming results obtained by microscopy-based quantification of SAC failure. An asterisk denotes $P < 0.001$ (1-way ANOVA with post-hoc Bonferroni's correction), $n = 3$ flow assays per FA genotype, and all bars represent mean values ± SEM.

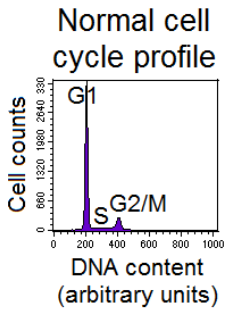
Genetic correction of primary cells from FA patients

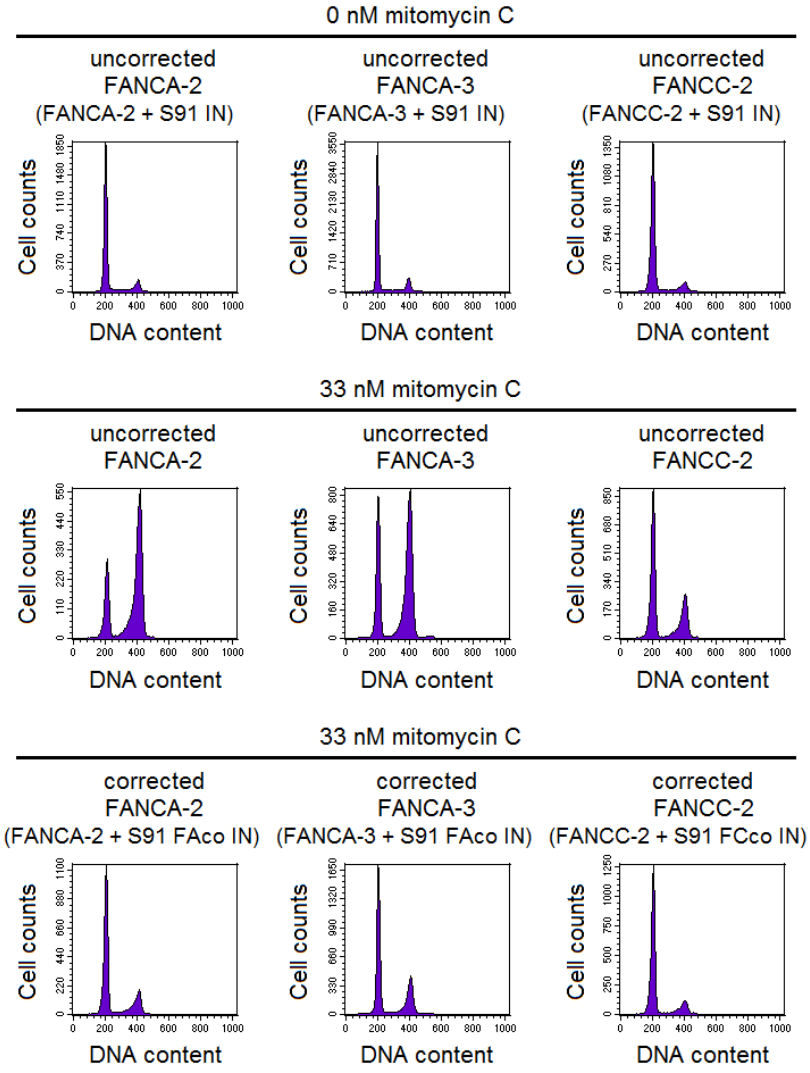
To generate isogenic FA patient cells and gene-corrected complementary cells, primary fibroblasts from an FA patient of the FANCA or FANCC subtype were transduced with either an empty lentiviral vector or a lentiviral vector containing the full-length, codon-optimized version of the FANCA or FANCC gene (Figure 3-6a). Two different FANCA patient fibroblast lines and one FANCC patient fibroblast line were successfully gene-corrected using two different vector systems, one containing a neomycin resistance cassette and the other containing a puromycin resistance cassette (Figure 3-6b). Neomycin or puromycin respectively was used to select for transduced cells (Figure 3-6a). Finally, to verify functional correction, the stably transduced cells were challenged with MMC and cell cycle analysis was performed via flow cytometry. Stable expression of the missing FANCA protein in primary FANCA-deficient fibroblasts rescued cell cycle block in response to mitomycin, which is known to result from loss of FA signaling (Figure 3-6c and d).


a
Empty control vector
Uncorrected FANCA (or FANCC) patient cells
Lentiviral transduction
Neomycin or puromycin selection
Primary FANCA (or FANCC) patient fibroblast cells
Vector containing full-length, codon-optimized FANCA (or FANCC) gene
Gene-corrected FANCA (or FANCC) patient cells


b
IRES-NEO (IN) cassette-containing vectors
IRES-PURO (IP) cassette-containing vectors
S91 IN
S91 FANCAco IN
S11 IP
S91 FANCAco IP
S91 IN
S91 FANCCco IN
S11 IP
S91 FANCCco IP

c


Normal cell cycle profile
G1
S
G2/M
Cell counts
DNA content (arbitrary units)


0 nM mitomycin C
uncorrected FANCA-2 (FANCA-2 + S91 IN)
uncorrected FANCA-3 (FANCA-3 + S91 IN)
uncorrected FANCC-2 (FANCC-2 + S91 IN)
33 nM mitomycin C
uncorrected FANCA-2
uncorrected FANCA-3
uncorrected FANCC-2
33 nM mitomycin C
corrected FANCA-2 (FANCA-2 + S91 FAco IN)
corrected FANCA-3 (FANCA-3 + S91 FAco IN)
corrected FANCC-2 (FANCC-2 + S91 FCco IN)
Cell counts
DNA content

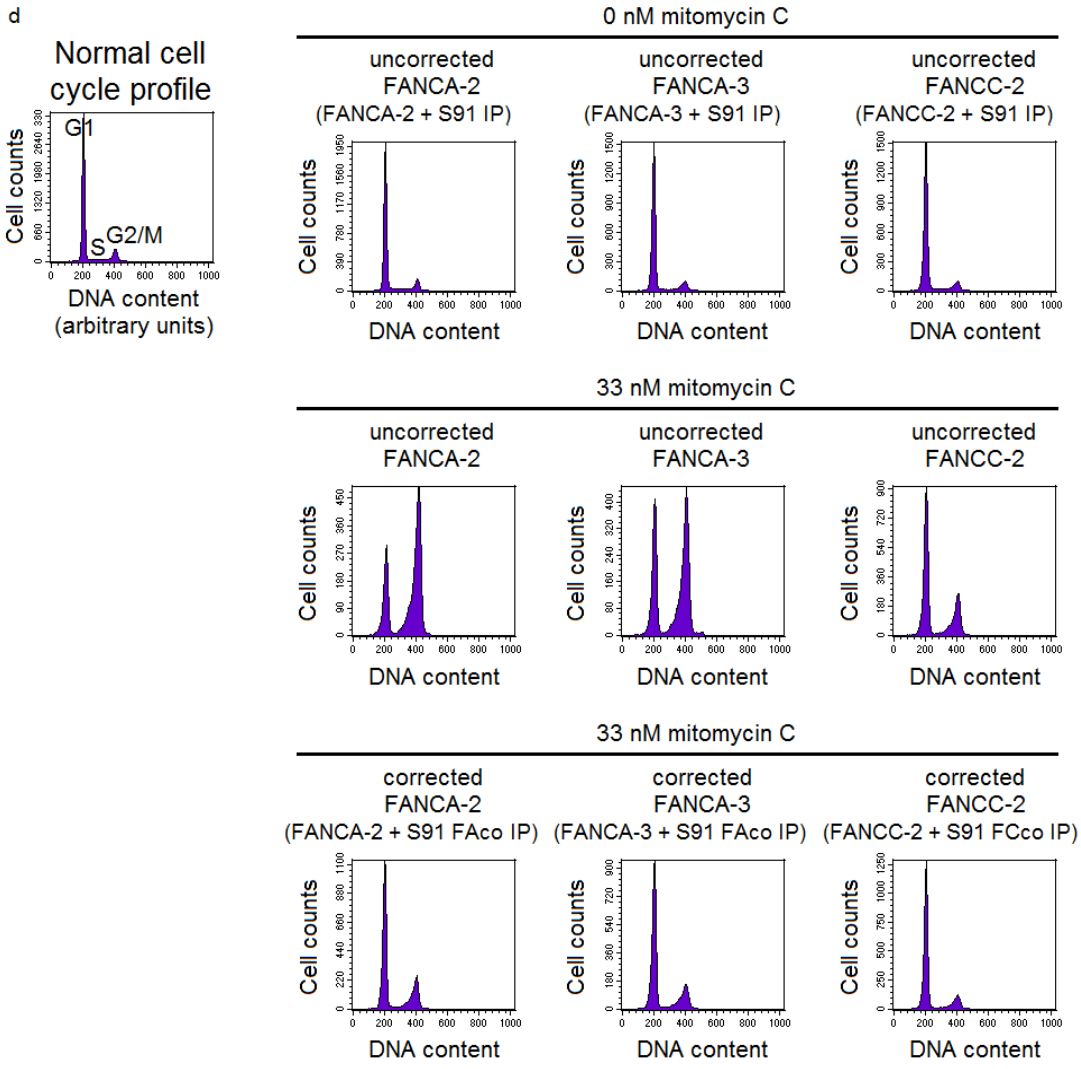

d
Normal cell cycle profile
G1
S
G2/M
Cell counts
DNA content (arbitrary units)
0 nM mitomycin C
uncorrected FANCA-2 (FANCA-2 + S91 IP)
uncorrected FANCA-3 (FANCA-3 + S91 IP)
uncorrected FANCC-2 (FANCC-2 + S91 IP)
33 nM mitomycin C
uncorrected FANCA-2
uncorrected FANCA-3
uncorrected FANCC-2
33 nM mitomycin C
corrected FANCA-2 (FANCA-2 + S91 FAco IP)
corrected FANCA-3 (FANCA-3 + S91 FAco IP)
corrected FANCC-2 (FANCC-2 + S91 FCco IP)
Cell counts
DNA content

Figure 3-6. Generation of functionally gene-corrected FANCA and FANCC patient fibroblasts. **a)** Schematic illustrating generation of gene-corrected FA patient fibroblasts. Stable ectopic expression of *FANCA* or *FANCC* in primary fibroblasts from FA patients of the FANCA and FANCC subtypes respectively was achieved via lentiviral transduction, followed by selection with neomycin or puromycin. **b)** Summary of vectors utilized in gene-correction of primary FANCA and FANCC patient fibroblasts. **c)** Demonstration of functional correction in response to MMC challenge for cells stably transduced with IRES-NEO cassette-containing vectors. **d)** Demonstration of functional correction in response to MMC challenge for cells stably transduced with IRES-PURO cassette-containing vectors. For **c and d,** cells were treated with 0 or 33 nM mitomycin C (MMC) for 72 hours. DNA was stained with propidium iodide, and cell cycle profiles were obtained via flow cytometry. MMC induces DNA interstrand crosslink damage which cannot be repaired in FA pathway-deficient cells, resulting in cell cycle arrest in late S phase. The resulting cell cycle block appears as a G2/M peak on cell cycle flow. In **c and d,** cell cycle block is observed for FANCA-deficient and FANCC-deficient fibroblasts treated with 33 nM MMC, while gene-correction via stable transduction with FANCA-expressing or FANCC-expressing vector results in ablation of the cell cycle block in response to MMC.

Genetic rescue of mitotic SAC activity in primary cells from FA patients

Previous experiments in siRNA-transfected HeLa cells and primary FA patient fibroblasts demonstrated that mitotic SAC failure occurs in the absence of an intact FA pathway. Since we observed weakened activity of the mitotic SAC in primary fibroblasts from FA patients, we wanted to know if genetic correction via stable expression of the mutated FA protein would correct the SAC defect in these cells. Importantly, stable ectopic expression of the mutated FANCA protein in primary fibroblasts from an FA patient of the FANCA subtype rescued the mitotic SAC (Figure 3-7). The SAC was tested in response to taxol challenge in an additional uncorrected and gene-corrected pair (FANCA-3 in Figure 3-6 above), and the same result was observed (data not shown).

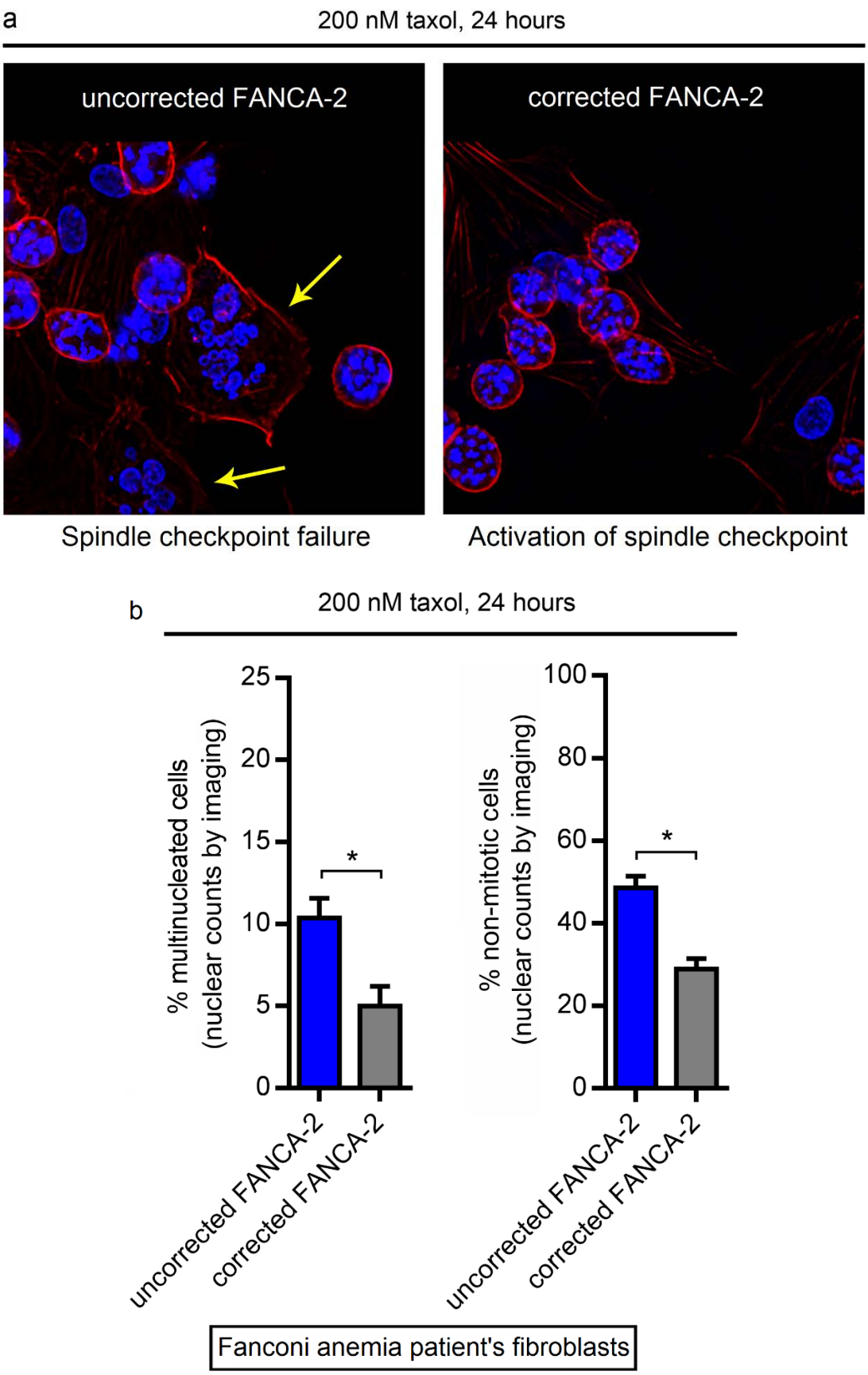

a
200 nM taxol, 24 hours
uncorrected FANCA-2
corrected FANCA-2
Spindle checkpoint failure
Activation of spindle checkpoint
b
200 nM taxol, 24 hours
% multinucleated cells (nuclear counts by imaging)
25
20
15
10
5
0
*
uncorrected FANCA-2
corrected FANCA-2
% non-mitotic cells (nuclear counts by imaging)
100
80
60
40
20
0
*
uncorrected FANCA-2
corrected FANCA-2
Fanconi anemia patient's fibroblasts

Figure 3-7. Ectopic expression of FANCA rescues the SAC defect in primary FANCA patient fibroblasts exposed to taxol. Uncorrected and gene-corrected primary FANCA patient fibroblasts were challenged with taxol for 24 hours, stained to label DNA and actin, and analyzed via microscopy. **a)** Representative images of taxol-challenged uncorrected and gene-corrected FANCA-deficient primary fibroblasts. Uncorrected FANCA fibroblasts fail to arrest in mitosis following taxol challenge and generate multinucleated cells (arrows), while isogenic gene-corrected control fibroblasts demonstrate appropriate prometaphase arrest following taxol exposure. Original magnification is ×200 (Applied Precision PersonalDV). **b)** Quantification of the microscopy-based results. In the graph of % multinucleated cells, the asterisk denotes $P = 0.0038$ (2-tailed *t* test), $n = 15$ microscopic fields, and bars represent mean values ± SEM. In the graph of % non-mitotic cells, the asterisk denotes $P < 0.0001$ (2-tailed *t* test), $n = 15$ microscopic fields, and bars represent mean values ± SEM.

Mitotic SAC failure in primary FANCA patient fibroblasts in response to nocodazole

As summarized in the introduction, taxol and nocodazole are mechanistically different chemotherapeutic agents targeting the mitotic spindle. Nocodazole, a microtubule destabilizing agent, promotes destruction of the mitotic spindle, resulting in virtually a complete absence of kinetochore-spindle fiber attachments. In contrast, taxol, a microtubule stabilizing agent, prevents remodeling of the mitotic spindle. The result is that some kinetochore-spindle fiber attachments may be possible in the presence of taxol. Unattached kinetochores generate the ‘on’ signal for the mitotic SAC, with the level of MAD2 reflecting the number of unattached kinetochores. As the percentage of attached kinetochores increases, the concentration of MAD2 at the kinetochore decreases and the strength of the SAC ‘on’ signal correspondingly decreases. Since nocodazole results in a greater number of unattached kinetochores than taxol, the SAC signal should be stronger in response to nocodazole than taxol. Thus, it follows that if the FA signaling network is essential for the mitotic SAC in response to taxol, the FA signaling network is also likely to be essential for the mitotic SAC in response to nocodazole. To assess the role of the FA pathway in the activity of the mitotic SAC in response to a microtubule destabilizing agent, primary FANCA-deficient cells were challenged with nocodazole and mitotic SAC failure was quantified.

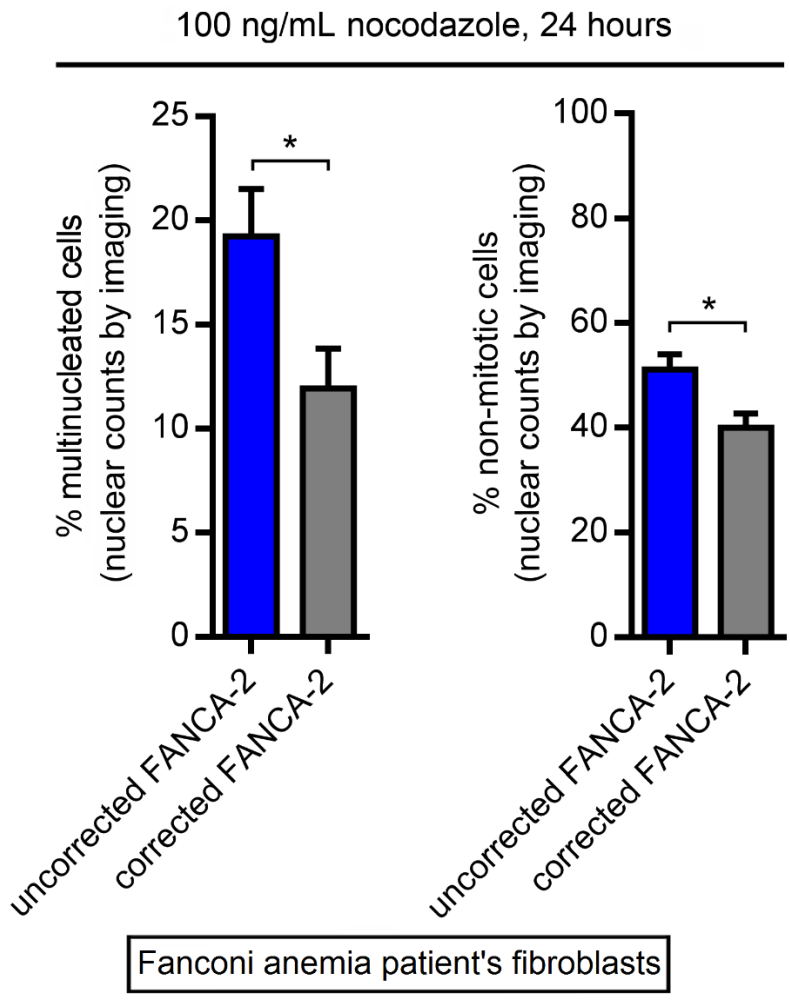


Figure 3-8. Primary FANCA-deficient patient fibroblasts exhibit weakened activity of the mitotic SAC in response to nocodazole. Similar to taxol-challenged cells, uncorrected and gene-corrected primary FANCA patient fibroblasts were challenged with nocodazole for 24 hours, stained to label DNA and actin, and analyzed via microscopy. The graphs shown represent the quantification of microscopy-based results. In the graph of % multinucleated cells, the asterisk denotes $P = 0.021$ (2-tailed t-test), $n = 15$ microscopic fields, and bars represent mean values ± SEM. In the graph of % non-mitotic cells, the asterisk denotes $P = 0.0096$ (2-tailed t-test), $n = 15$ microscopic fields, and bars represent mean values ± SEM.

FANCA shRNA knockdown and characterization of mitotic SAC phenotype in ex vivo-cultured CD34+ cells

Bone marrow failure, AML, and MDS are the major causes of morbidity and mortality in FA. Since FA patients frequently develop aneuploidy-associated hematologic malignancies, we wanted to determine whether the mitotic SAC contributes to genomic instability in phenotypic hematopoietic stem and progenitor cells. Thus, we tested whether FANCA controls the SAC in primary hematopoietic cells.

To generate FANCA-knockdown primary hematopoietic stem and progenitor cells, CD34+ cells were isolated from umbilical cord blood, transduced with FANCA shRNA or scrambled control shRNA, and sorted for GFP+ transduced cells. The FANCA shRNA used in this experiment has previously been validated by demonstrating increased MMC hypersensitivity and ablation of FANCD2 monoubiquitination in transduced cells (Z. Sun, D. W. Clapp, H. Hanenberg, unpublished data). Since many CD34+ cells are quiescent, BrdU was used to label the cycling cells. Then, the SAC was challenged using taxol, and cells were immunostained for BrdU and phospho-histone H3 to label cycling and mitotic cells respectively and stained with Hoechst 33342 to label DNA. Finally, data was collected using an Amnis ImageStreamX Mark II imaging flow cytometer, which performs multicolor confocal imaging at the single cell level (Figure 3-9a), and data was analyzed using Amnis IDEAS software.

In this experiment, G2, M, and SAC failure cells could be distinguished from G0, G1, and S phase cells based on their DNA content and BrdU status.

Cells which have completed S phase DNA replication have 4N DNA content and are BrdU+. Phospho-H3 staining enabled the identification of mitotically arrested cells. However, imaging was necessary to identify multinucleated, SAC failure cells, which have the same BrdU status and DNA content as pre-mitotic G2 cells (Figure 3-9b). When multinucleated cells were quantified, FANCA-knockdown primary CD34+ cells exhibited weakened SAC activity compared with negative control cells, as evidenced by a significant increase in the percentage of multinucleated cells following exposure to taxol (Figure 3-9c and d).

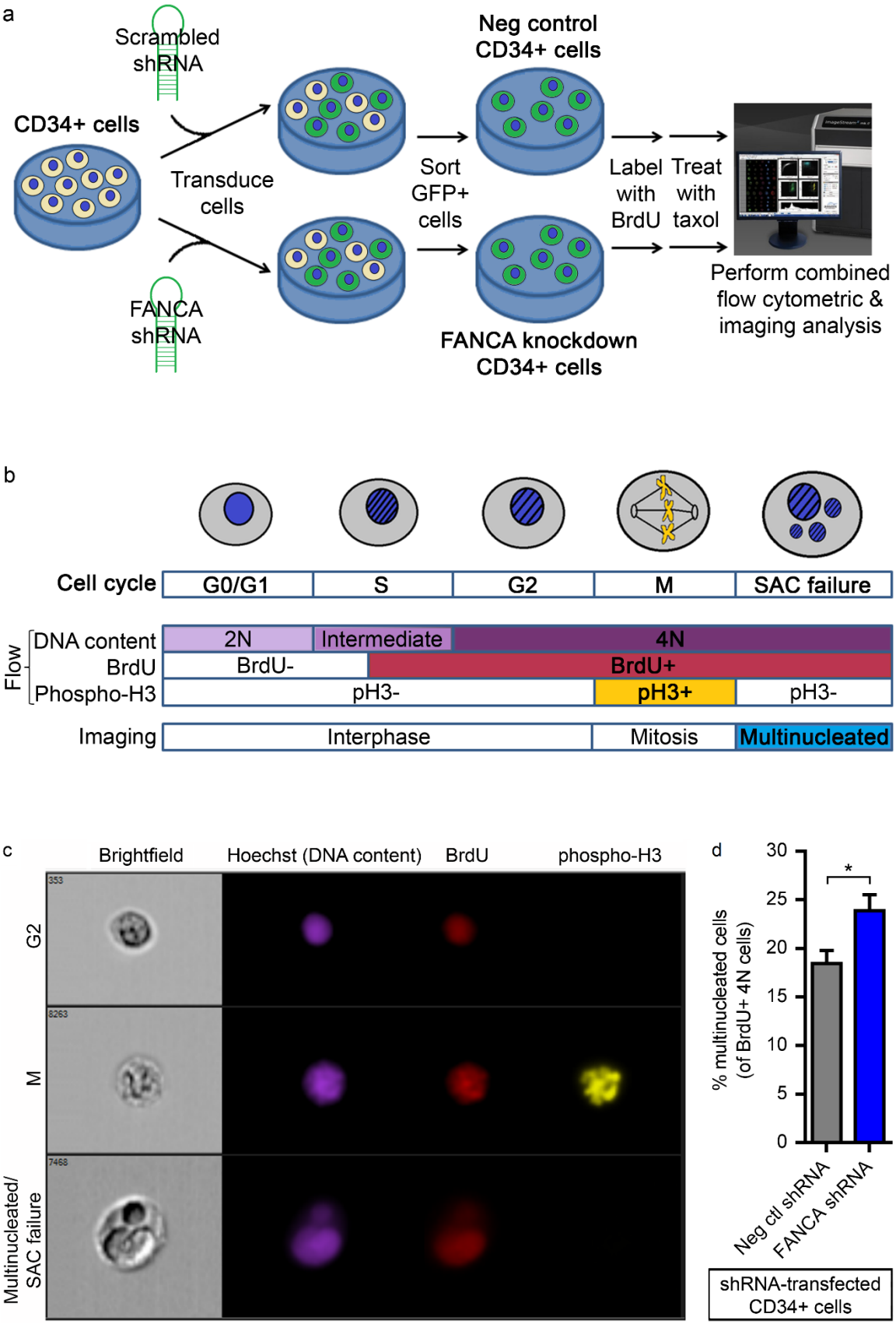

a
Scrambled shRNA
CD34+ cells
Transduce cells
FANCA shRNA
Neg control CD34+ cells
Sort GFP+ cells
FANCA knockdown CD34+ cells
Label with BrdU
Treat with taxol
Perform combined flow cytometric & imaging analysis
b
Cell cycle
G0/G1
S
G2
M
SAC failure
Flow
DNA content
BrdU
Phospho-H3
2N
Intermediate
4N
BrdU-
BrdU+
pH3-
pH3+
pH3-
Imaging
Interphase
Mitosis
Multinucleated
c
Brightfield
Hoechst (DNA content)
BrdU
phospho-H3
G2
M
Multinucleated/ SAC failure
d
% multinucleated cells (of BrdU+ 4N cells)
30
25
20
15
10
5
0
*
Neg ctl shRNA
FANCA shRNA
shRNA-transfected CD34+ cells

Figure 3-9. Analysis of mitotic SAC activity in FANCA-deficient primary CD34+ cells demonstrates that FANCA is essential for the mitotic SAC in human hematopoietic cells. a) Schematic of microscopy-coupled flow cytometry-based assessment of mitotic SAC activity in primary CD34+ cells. CD34+ cells were transduced with shRNA, sorted for GFP+ transduced cells, labeled with BrdU, and treated with taxol prior to analysis using a flow cytometer which performs confocal imaging of each cell. Hoechst 33342 was used to label DNA, and immunostaining for BrdU and phospho-histone H3 were used to label cycling and mitotic cells respectively. **b)** Microscopy-coupled flow cytometry allows quantification of SAC failure in cycling $CD34^+$ cells. Mitotic cells are phospho-histone H3-positive, and SAC failure cells are multinucleated. **c)** Representative images of taxol-challenged FANCA-knockdown primary CD34+ cells. When BrdU-positive cells with 4N DNA content were gated, the observed phenotypes included interphase cells (G2), prometaphase cells (mitotic SAC arrest), and multinucleated interphase cells (SAC failure). Original magnification is ×400 (Amnis ImageStreamX Mark II). **d)** Quantification of SAC failure in cycling FANCA shRNA-transduced $CD34^+$ cells. The asterisk indicates $P = 0.029$ (2-tailed t-test), $n = 6$ assays, and both bars represent mean values ± SEM.

Hypersensitivity to drugs targeting spindle assembly in primary FANCA patient fibroblasts

We wanted to know if the weakened SAC we found in previous experiments results in a hypersensitivity to spindle poisons such as taxol in FA pathway-deficient primary cells. Thus, a clonogenic assay was performed on primary uncorrected and gene-corrected FANCA-deficient fibroblasts (FANCA-2 in Figure 3-6 above) to assess the effect of taxol on survival and proliferation in the presence and absence of FANCA. Uncorrected FANCA-deficient fibroblasts demonstrated decreased clonogenic growth in the presence of low doses of taxol compared with isogenic gene-corrected control fibroblasts, indicating that loss of FANCA results in hypersensitivity to taxol. This assay was repeated on an additional uncorrected and gene-corrected pair (FANCA-3 in Figure 3-6 above), and the same result was observed (data not shown).

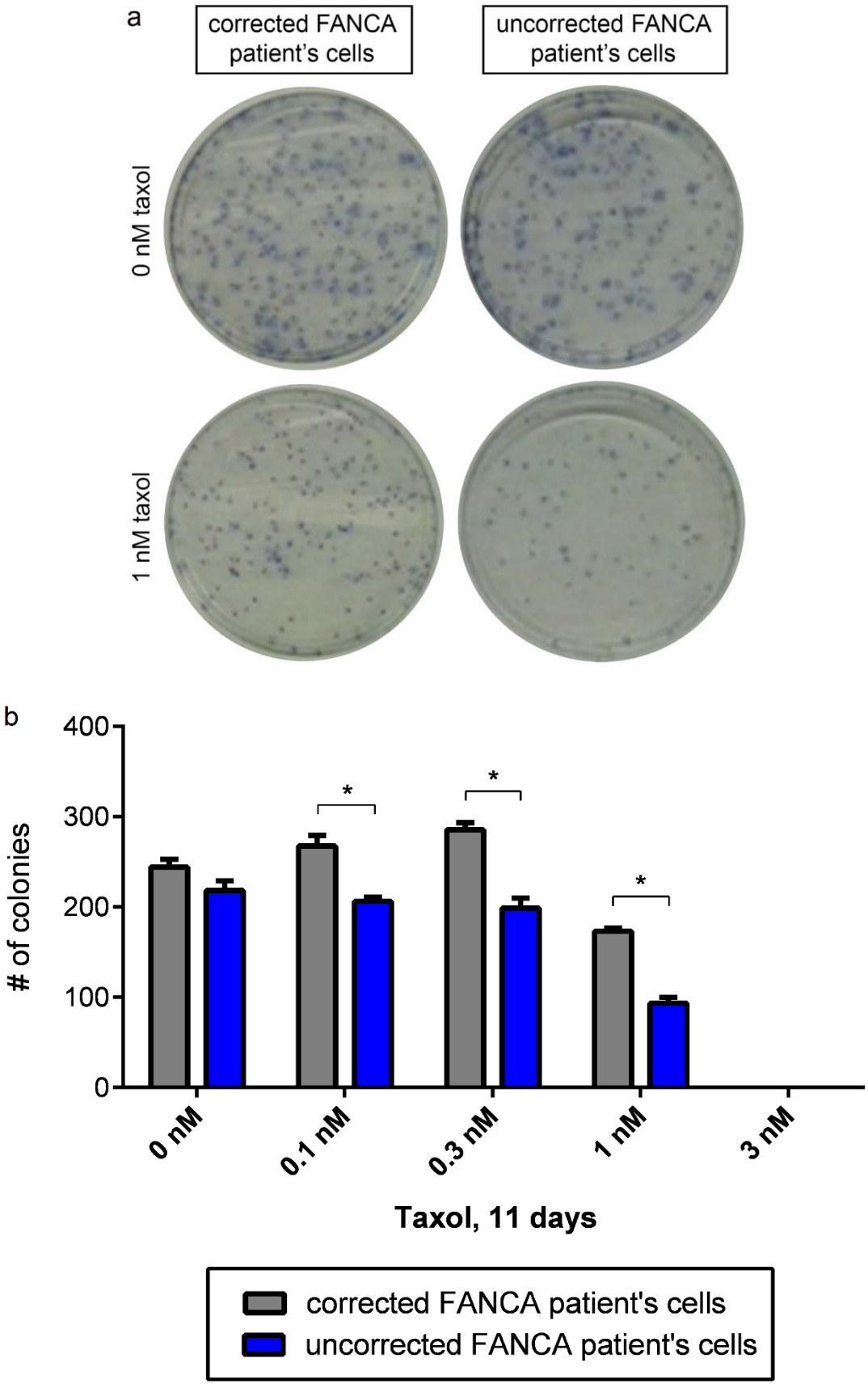
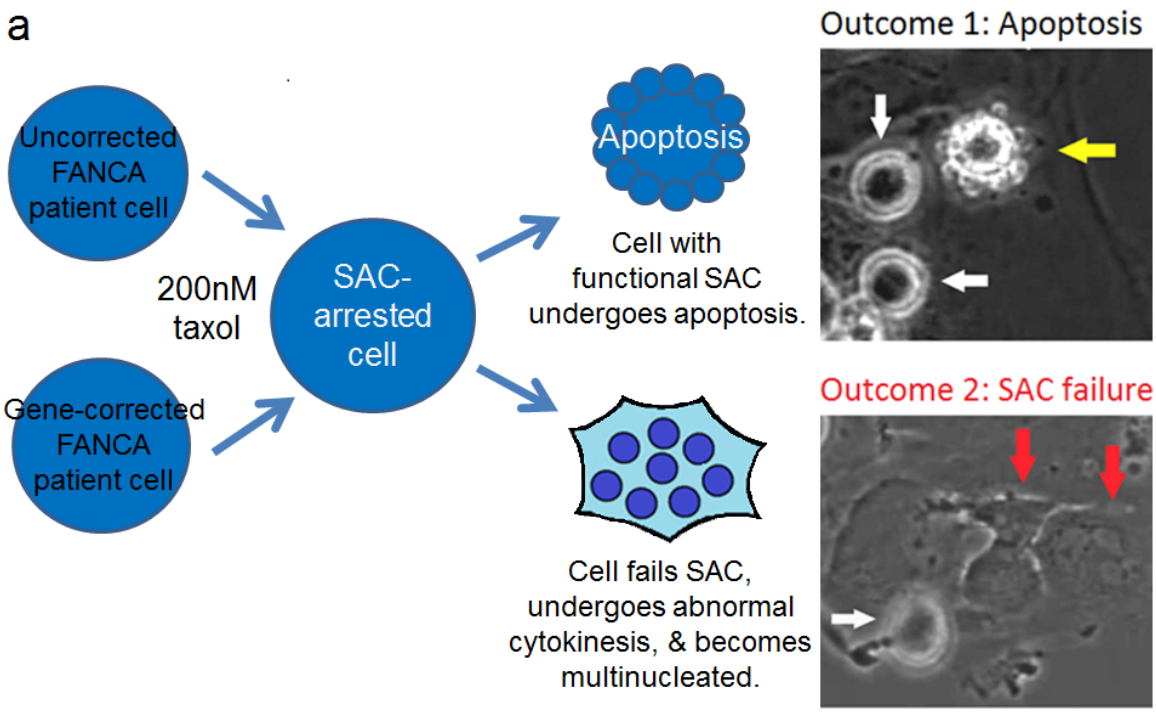
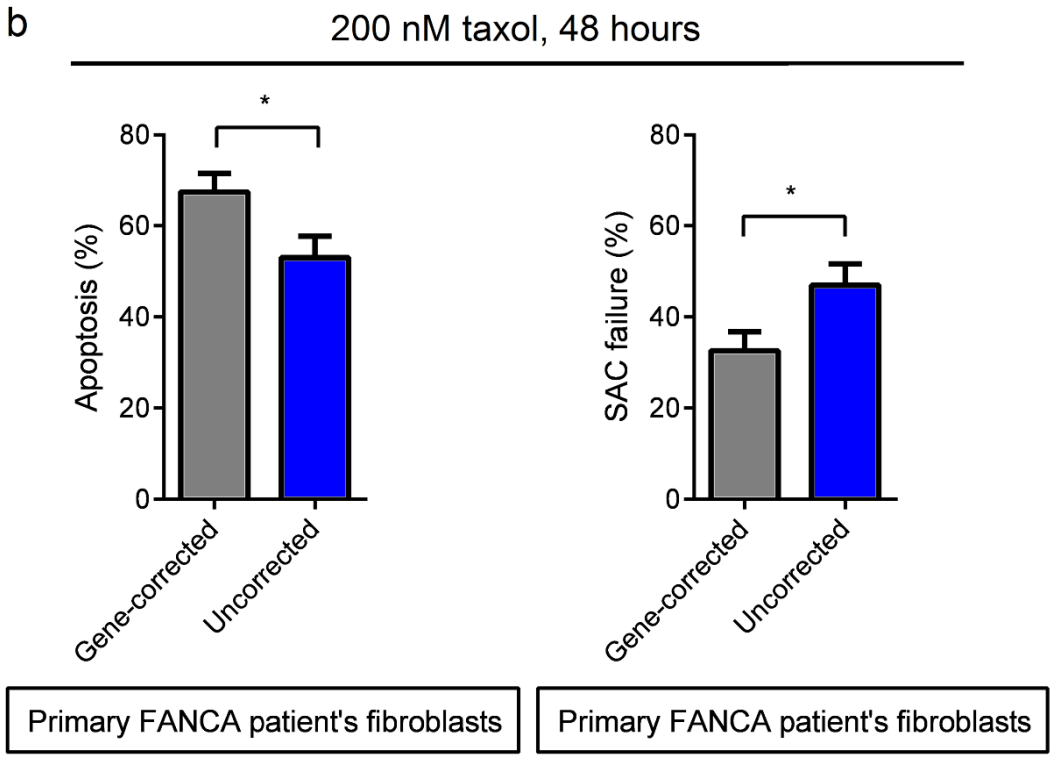

a
corrected FANCA patient's cells
uncorrected FANCA patient's cells
0 nM taxol
1 nM taxol
b
# of colonies
400
300
200
100
0
*
*
*
0 nM
0.1 nM
0.3 nM
1 nM
3 nM
Taxol, 11 days
corrected FANCA patient's cells
uncorrected FANCA patient's cells

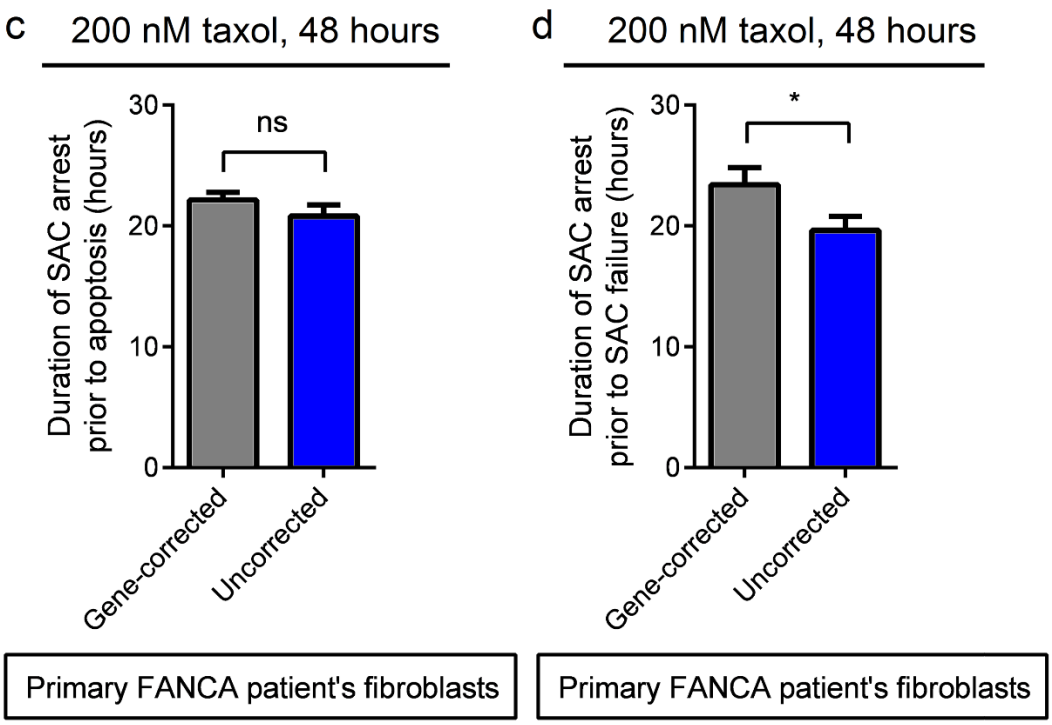
Figure 3-10. Clonogenic assay demonstrates that primary FANCA patient fibroblasts are hypersensitive to taxol. To assess the effect of taxol on survival and proliferation in the presence and absence of FANCA, uncorrected and isogenic gene-corrected FANCA patient cells were plated at 500 cells per 10-cm dish and cultured in the presence of 0, 0.1, 0.3, 1, or 3 nM taxol for 11 days. After staining with methylene blue, colonies were manually quantified. **a)** Representative examples of stained clonogenic assay plates. **b)** Quantification of results. Taxol concentration and FANCA status were considered 2 independent factors. Taxol concentration, FANCA status, and the interaction between the two factors resulted in significant differences by 2-way ANOVA ($P < 0.0001$). Asterisks indicate $P < 0.0001$ for comparisons between uncorrected and gene-corrected cells at 0.1, 0.3, and 1 nM taxol concentrations respectively by 2-way ANOVA with post-hoc Tukey's multiple comparison test. At 0 and 3 nM taxol, uncorrected and gene-corrected FANCA patient fibroblasts were not significantly different. $n = 6$ assays, and bars represent mean values ± SEM.

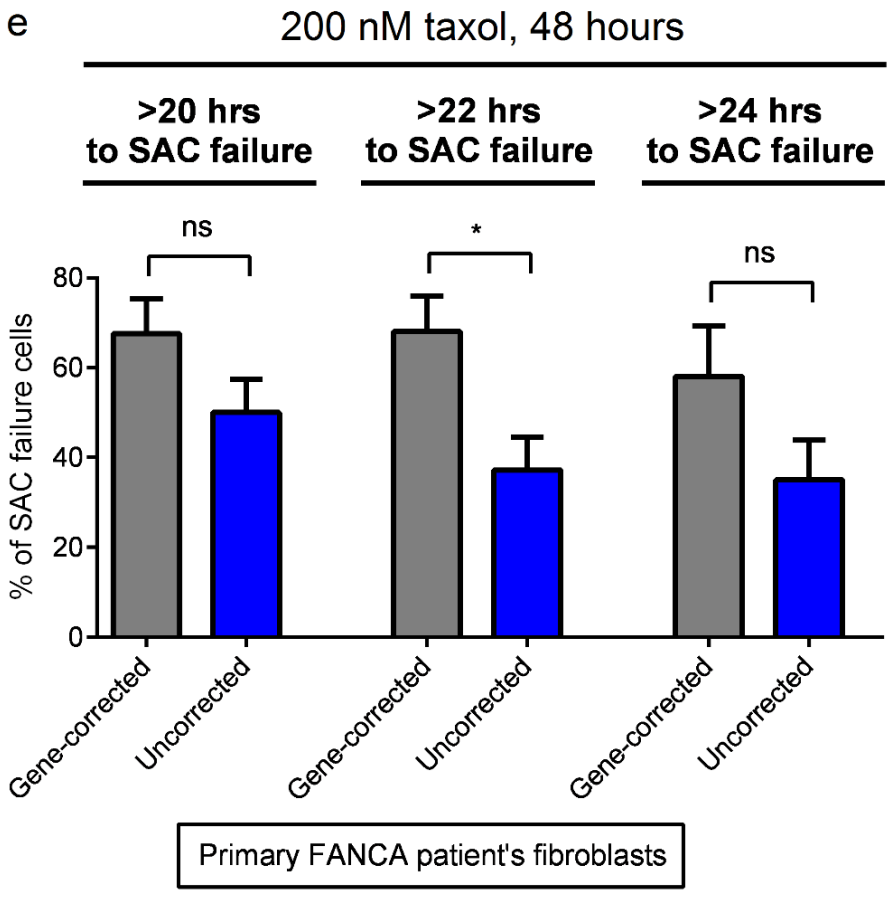
Systematic assessment of mitotic SAC failure in primary FANCA patient fibroblasts by video microscopy

In order to quantify the duration of SAC arrest and directly visualize the outcome of SAC arrest in FA pathway-deficient cells, time-lapse microscopy of taxol-challenged primary FANCA-deficient fibroblasts was performed. The outcome of prolonged SAC arrest may be either apoptosis or SAC failure. When the SAC is functioning properly, cells will initiate apoptosis in response to prolonged SAC arrest. The coupling of apoptosis to SAC arrest is an essential mechanism for the prevention of aneuploidy in daughter cells because the elimination of dividing cells which are unable to complete the formation of kinetochore-spindle fiber attachments prevents the development of lagging chromosomes which may segregate to the wrong daughter cell. As previously described, when the activity of the SAC is weakened, a cell may initiate anaphase in the presence of lingering unattached kinetochores. In other words, the cell may have a lower threshold for satisfaction of the SAC. When the SAC fails and anaphase initiation occurs prematurely, the result may be mis-segregation of chromosomes, contributing to genomic instability.


a
Uncorrected FANCA patient cell
Gene-corrected FANCA patient cell
200nM taxol
SAC-arrested cell
Apoptosis
Cell with functional SAC undergoes apoptosis.
Cell fails SAC, undergoes abnormal cytokinesis, & becomes multinucleated.
Outcome 1: Apoptosis
Outcome 2: SAC failure


b
200 nM taxol, 48 hours
Apoptosis (%)
SAC failure (%)
0
20
40
60
80
*
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts
Primary FANCA patient's fibroblasts


c
200 nM taxol, 48 hours
Duration of SAC arrest prior to apoptosis (hours)
ns
30
20
10
0
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts
d
200 nM taxol, 48 hours
Duration of SAC arrest prior to SAC failure (hours)
*
30
20
10
0
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts


e
200 nM taxol, 48 hours
>20 hrs to SAC failure
>22 hrs to SAC failure
>24 hrs to SAC failure
% of SAC failure cells
ns
*
ns
80
60
40
20
0
Gene-corrected
Uncorrected
Gene-corrected
Uncorrected
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts

f

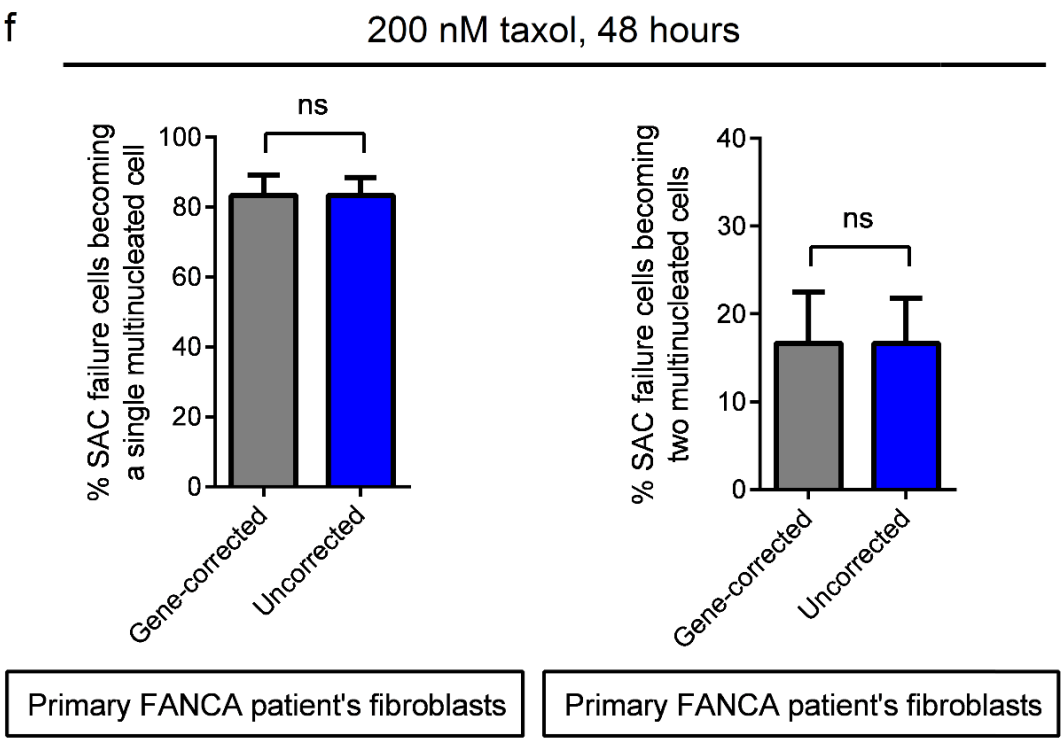

200 nM taxol, 48 hours
ns
% SAC failure cells becoming a single multinucleated cell
100
80
60
40
20
0
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts
ns
% SAC failure cells becoming two multinucleated cells
40
30
20
10
0
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts

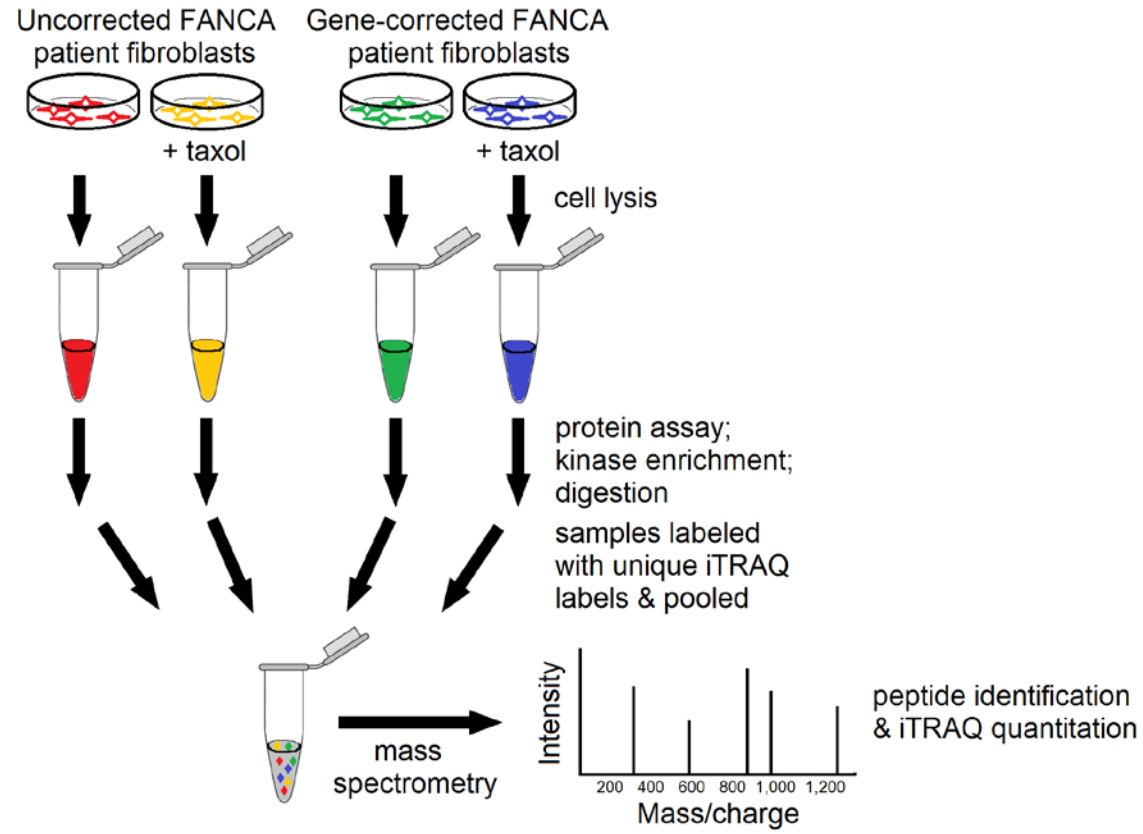
Figure 3-11. Video microscopy of taxol-challenged primary FANCA patient fibroblasts elucidates the role of FANCA in the activity of the mitotic SAC. Primary FANCA-deficient fibroblasts and isogenic gene-corrected control fibroblasts were challenged with 200 nM taxol and video microscopy was performed for 48 hours in order to quantify the duration of SAC arrest and record the outcome of prolonged SAC arrest. In response to prolonged SAC arrest, taxol-challenged fibroblasts either exhibited the normal apoptotic response or exhibited SAC failure. SAC failure was visualized by initiation of cytokinesis followed by progression to interphase. Cells exhibiting SAC failure became one or two interphase multinucleated cells reflecting cytokinesis failure or completion respectively. **a)** Schematic of experimental design and representative images. Original magnification is ×200 (Nikon BioStation IM-Q). White arrows indicate SAC arrest, red arrows indicate SAC failure, and the yellow arrow indicates apoptosis. Total n=144-171 cells per genotype. At the end of quantified videos, each cell exhibited one of three outcomes—apoptosis, SAC failure, or SAC arrest through the end of the video. Cells remaining arrested at the end of the videos were omitted from analysis. **b)** Compared with isogenic gene-corrected control fibroblasts, a decreased percentage of primary FANCA-deficient fibroblasts initiate apoptosis and an increased percentage of primary FANCA-deficient fibroblasts exhibit SAC failure as the outcome of prolonged mitotic SAC arrest induced by challenge with 200 nM taxol (P=0.0215). n=115-129 cells per genotype. **c)** Taxol-challenged primary FANCA-deficient fibroblasts and gene-corrected control fibroblasts remain arrested at the mitotic SAC for similar lengths of time prior to initiation of apoptosis (P=0.2377). n=48-72 cells per genotype. **d)** For cells exhibiting SAC failure in response to taxol challenge, primary FANCA-deficient fibroblasts maintain SAC arrest for a shorter duration prior to the initiation of cytokinesis than do gene-corrected control fibroblasts (P=0.0396). n=37-46 cells per genotype. **e)** For cells exhibiting SAC failure following taxol challenge, primary FANCA-deficient fibroblasts fail to maintain SAC arrest for as long as control fibroblasts. An approximately two-fold decrease in the percentage of cells maintaining the SAC for >22 hours was observed for FANCA-deficient fibroblasts compared with gene-corrected control fibroblasts (P=0.0052). While fewer FANCA-deficient fibroblasts maintained SAC arrest for >20 hours and >24 hours compared with control fibroblasts, the observed differences were not statistically significant (P=0.1098 and P=0.1028 respectively). n=37-46 cells per genotype. Differences in the duration of SAC arrest between uncorrected and gene-corrected primary FANCA patient fibroblasts were analyzed at thresholds of 20, 22, and 24 hours because the average durations of SAC arrest were 19.64 hours (uncorrected) and 23.41 hours (corrected). **f)** Cells which exhibit SAC failure and initiate cytokinesis may subsequently fail to complete cytokinesis, resulting in a single multinucleated daughter cell, or may successfully complete cytokinesis, resulting in two daughter cells. Following taxol challenge, similar numbers of SAC-arrested FANCA-deficient fibroblasts and isogenic gene-corrected control fibroblasts progressed to each endpoint (P=>0.9999). n=37-46 cells per genotype. The majority of cells which failed to maintain SAC arrest subsequently failed to complete cytokinesis.

Disruption of FANCA results in altered expression and post-translational modification of mitotic SAC regulators at the proteome level

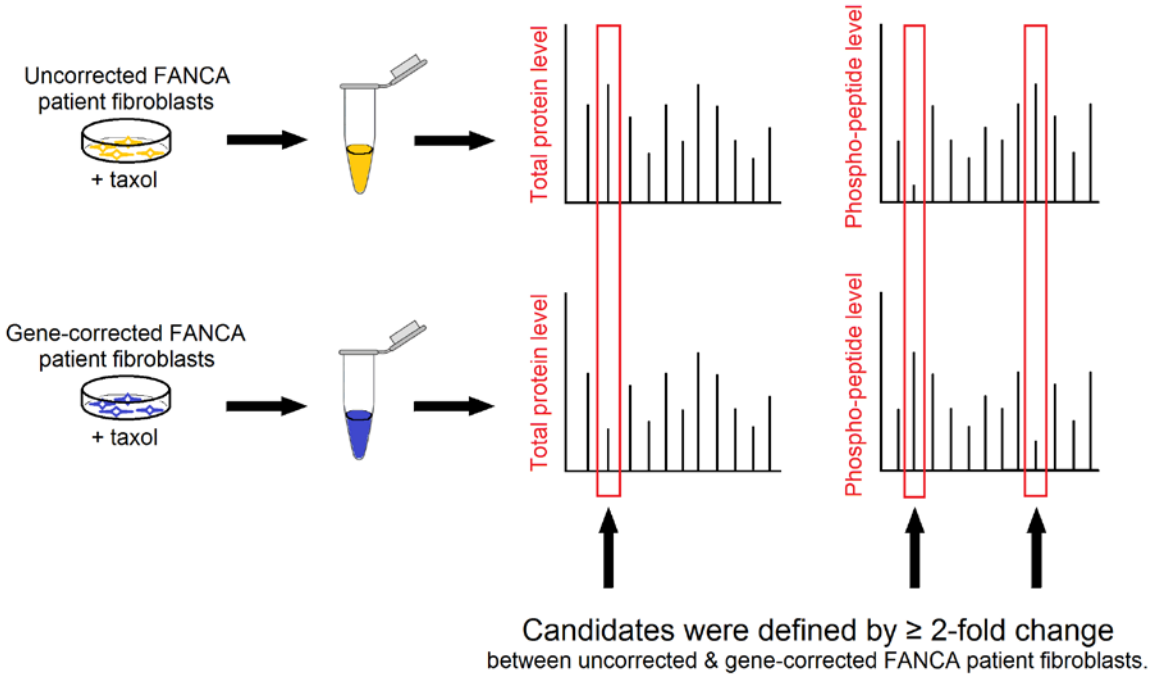
Finally, to identify mitotic signaling pathways affected by the loss of FANCA, we performed a mass spectrometry-based screen of the proteome and phospho-proteome in primary FANCA patient-derived fibroblasts. Primary FANCA-deficient fibroblasts and their gene-corrected complement were challenged with 1 nM taxol for 9 days. The concentration and duration of taxol challenge were determined based on the results of the clonogenic assay (Figure 3-10). The total protein levels and phospho-peptide levels were quantified by mass spectrometry. Candidates were identified based on a two-fold or greater change in the quantified level of total or phospho-protein in the primary FANCA-deficient fibroblasts compared with their gene-corrected complement.

In taxol-challenged primary FANCA-deficient fibroblasts, a number of candidates were identified which are known to regulate mitosis, apoptosis, and DNA damage repair, including a number of candidates not previously linked to FA. In untreated and MMC-challenged controls, several proteins which are known to interact with the FA pathway in DNA interstrand crosslink repair were identified as candidates, validating the experimental approach. Two candidates which are known oncoproteins, mitotic regulators, and targets of the mitotic kinase Aurora A at the centrosome (BRCA1 and SKI) have been validated by immunoblotting, and we are working to validate additional candidates which are known regulators of the mitotic SAC.

a


Uncorrected FANCA patient fibroblasts
Gene-corrected FANCA patient fibroblasts
+ taxol
+ taxol
cell lysis
protein assay; kinase enrichment; digestion
samples labeled with unique iTRAQ labels & pooled
mass spectrometry
Intensity
200 400 600 800 1,000 1,200
Mass/charge
peptide identification & iTRAQ quantitation

b


Uncorrected FANCA patient fibroblasts
+ taxol
Gene-corrected FANCA patient fibroblasts
+ taxol
Total protein level
Phospho-peptide level
Total protein level
Phospho-peptide level
Candidates were defined by ≥ 2-fold change
between uncorrected & gene-corrected FANCA patient fibroblasts.

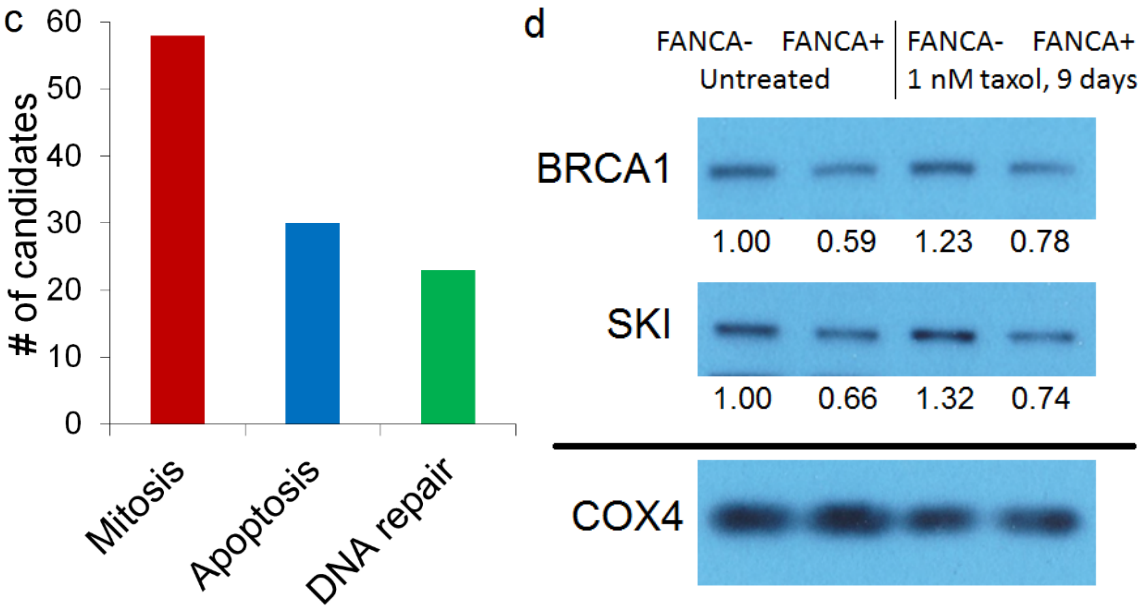


Figure 3-12. Altered expression and post-translational modification of mitotic regulators in primary FANCA patient fibroblasts. a) Schematic of the mass spectrometry-based screen. **b)** Schematic depicting how candidates were defined. **c)** Graph indicating the total number of candidates related to mitosis, apoptosis, and DNA repair which were identified in the analysis of taxol-challenged cells. **d)** Immunoblots validating the two mitosis-related candidates BRCA1 and SKI. For both untreated and taxol-challenged cells, increased expression of BRCA1 and SKI was observed in primary FANCA-deficient patient fibroblasts, compared with gene-corrected control fibroblasts. COX4 was used as a loading control. Three independent experiments were performed, and all results showed similarly increased BRCA1 and SKI in FANCA-deficient fibroblasts, compared with gene-corrected control fibroblasts, in both untreated and 1 nM taxol-challenged cells cultured for nine days. Representative immunoblots are shown, and the numbers beneath the immunoblots represent the relative expression level quantified by densitometry, normalized to COX4 loading control. Untreated, uncorrected FANCA patient fibroblasts were arbitrarily assigned to a value of 1.

CHAPTER FOUR

THE FA SIGNALING NETWORK IS ESSENTIAL FOR CENTROSOME MAINTENANCE AND PROPER EXECUTION OF UNPERTURBED MITOSIS

Introduction

Chapter three presented our discovery that the human FA signaling network is essential for the activity of the mitotic SAC, which protects genomic integrity by regulating chromosome segregation. Chapter four builds on this discovery by systematically examining the effects of unperturbed mitosis in FA pathway-deficient cells. As summarized in the previous chapter, video microscopy of taxol-challenged fibroblasts confirmed that loss of function mutations in *FANCA* result in a weakened SAC, evidenced by decreased duration of SAC arrest and more frequent SAC failure. Earlier initiation of chromosome segregation in SAC-arrested FA pathway-deficient cells may directly lead to aneuploidy either through the generation of anaphase lagging chromosomes or through the failure of cytokinesis.

Since the strength of the SAC activity ‘on’ signal generally correlates to the number of unattached kinetochores, weakening of the mitotic SAC may result in the initiation of anaphase chromosome segregation in the presence of one or more lingering unattached kinetochores. When anaphase is initiated prematurely, sister chromatids with unattached kinetochores or abnormally attached kinetochores can become anaphase lagging chromosomes which segregate to the incorrect daughter cell and form a micronucleus.

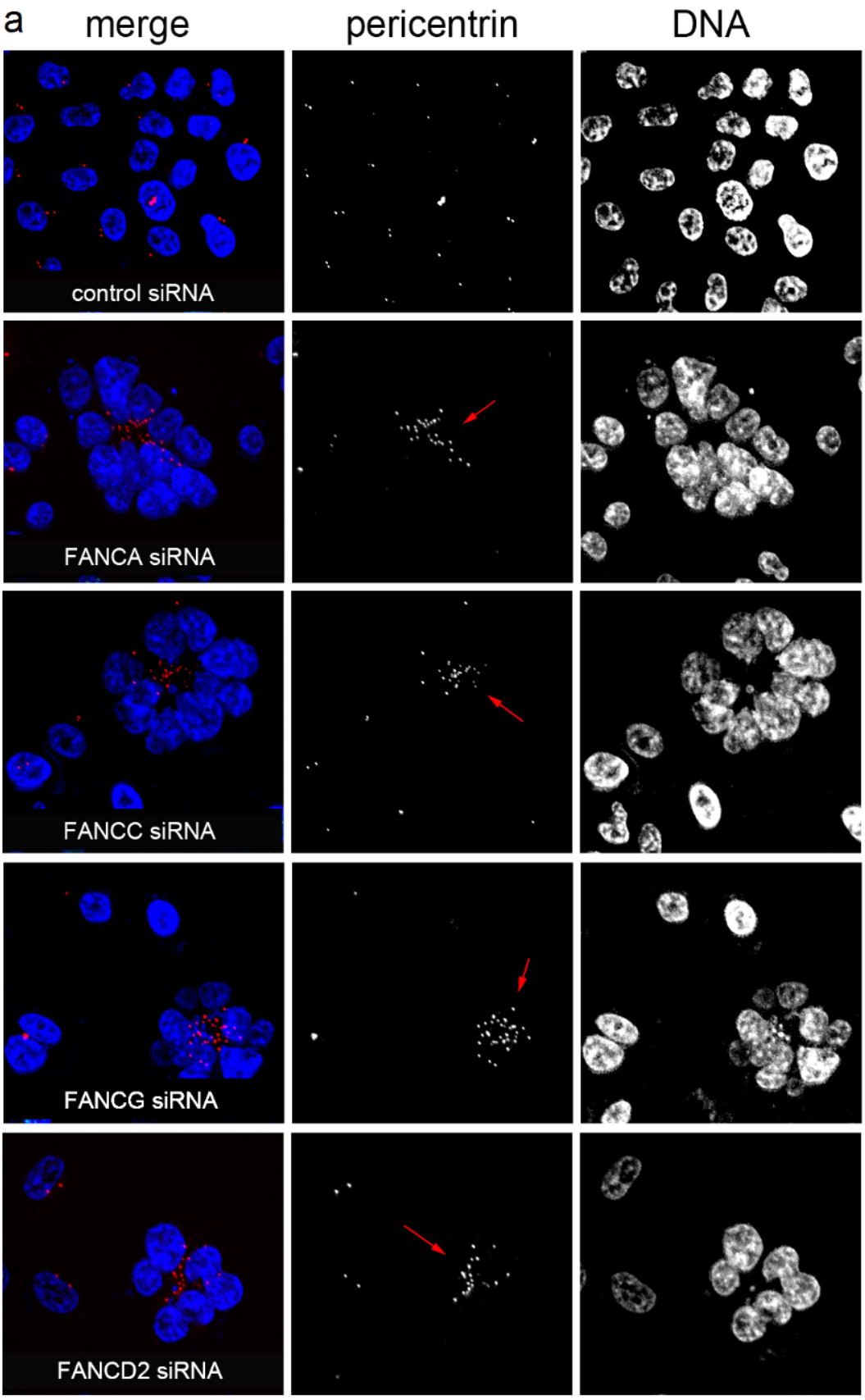
Video microscopy of taxol-challenged fibroblasts further confirmed that SAC failure is frequently followed by cytokinesis failure in taxol-challenged fibroblasts, regardless of FANCA status. Cytokinesis failure results in a 4N cell which may be binucleated or multinucleated. Additionally, cytokinesis failure generates a G1 cell with two centrosomes. In the next round of the cell cycle, the completion of centrosome replication during S phase will inherently result in supernumerary centrosomes in a cell which has previously experienced cytokinesis failure.

The experiments in chapter three utilized challenge with spindle drugs to assess the activity of the mitotic SAC. The experiments in the current chapter examine the results of mitosis which is allowed to occur in the absence of chemical perturbation. We hypothesized that a weakened mitotic SAC would result in accelerated progression through the early phases of mitosis and that aneuploidy and supernumerary centrosomes would occur as a result of unperturbed mitosis in FA pathway-deficient cells. To determine whether aneuploidy and supernumerary centrosomes develop as a result of unperturbed mitosis in FA pathway-deficient cells, we performed RNAi-based experiments and studies utilizing primary cells from FA patients. To determine whether the timing of unperturbed mitosis is accelerated or notable phenotypic defects exist in mitosis or cytokinesis in the absence of FANCA function, phase contrast video microscopy of unperturbed mitosis was performed in primary fibroblasts from an FA patient of the FANCA subtype.

Results

RNAi screen of FA proteins for essential role in centrosome maintenance

To determine whether the FA proteins are essential for the maintenance of normal numbers of centrosomes (one or two centrosomes per cell based on cell cycle status), HeLa cells were transfected with validated siRNAs against individual FA gene products as indicated (see Figure 3-2 for validation of siRNAs by immunoblotting). Cells were grown in the absence of spindle poisons for 72 hours and then fixed. Next, immunofluorescence staining of endogenous pericentrin (a known centrosomal protein) was performed, followed by deconvolution microscopy and manual quantification of the acquired images. HeLa cells transfected with siRNAs against thirteen individual FA genes spontaneously accumulated extra centrosomes and became multinucleated.


a
merge
pericentrin
DNA
control siRNA
FANCA siRNA
FANCC siRNA
FANCG siRNA
FANCD2 siRNA

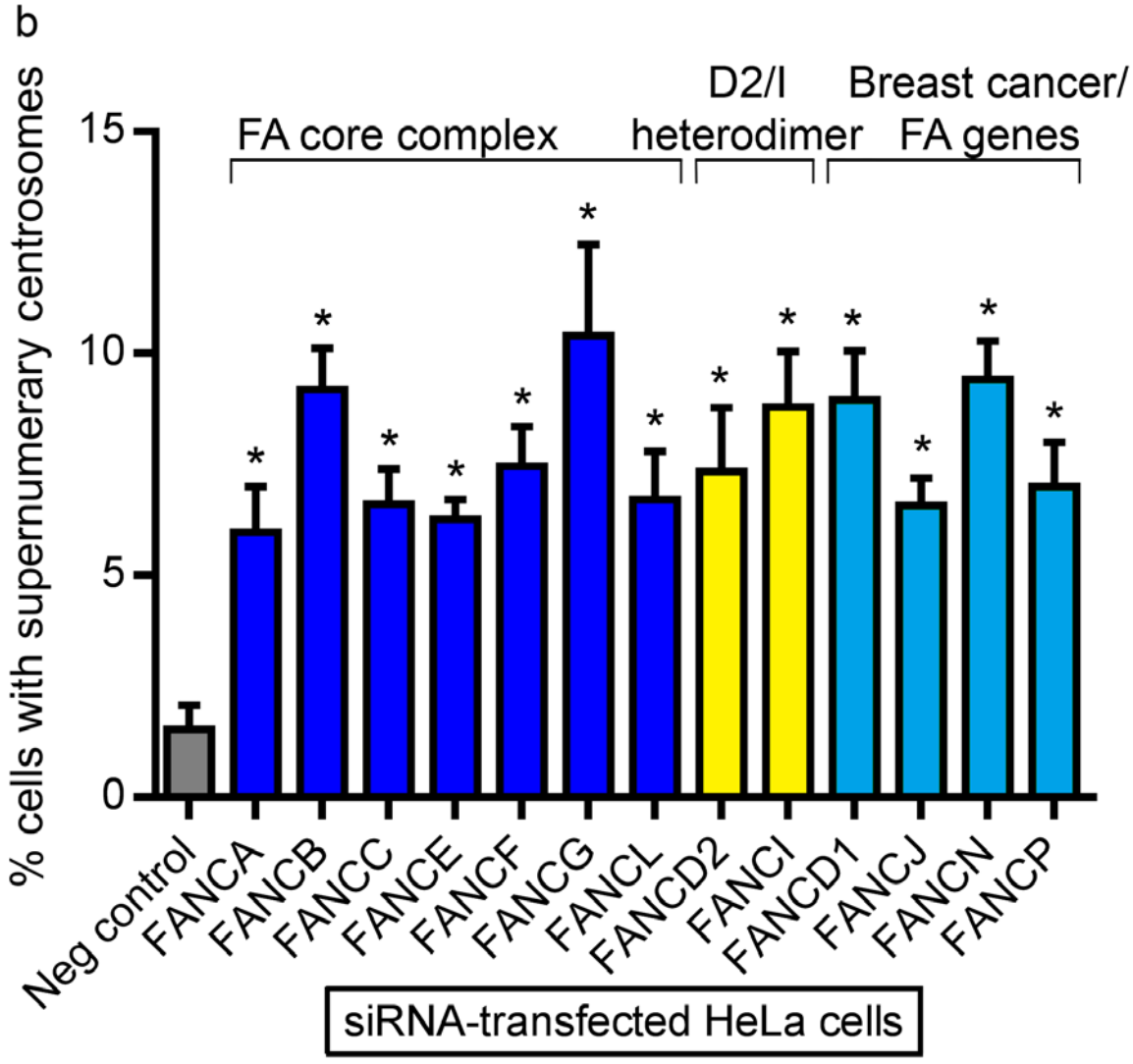
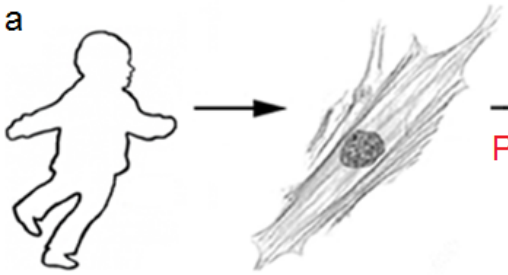

b
% cells with supernumerary centrosomes
15
10
5
0
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
Neg control
FANCA
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1
FANCJ
FANCN
FANCP
siRNA-transfected HeLa cells

Figure 4-1. Quantification of centrosomes in siRNA-transfected HeLa cells reveals a role for the FA signaling network in centrosome maintenance. RNAi knockdown of FA pathway gene products leads to spontaneous accumulation of supernumerary centrosomes and spontaneous multinucleation in HeLa cells. **a)** Representative images are shown for cells transfected with negative control siRNA or siRNA against an individual FA protein as indicated. Cells transfected with negative control siRNA have normal numbers of centrosomes and normal nuclear architecture. Cells transfected with siRNA against an FA gene product accumulate supernumerary centrosomes and undergo multinucleation. Red arrows indicate clusters of supernumerary centrosomes. **b)** Quantification of microscopy-based results. An increased percentage of cells with abnormal centrosome counts results from RNAi knockdown of thirteen FA pathway gene products. Asterisks indicate $P < 0.05$ compared with negative control (1-way ANOVA with post-hoc Bonferroni's correction), $n = 10$ microscopic fields, and all bars represent mean values ± SEM. Original magnification is ×200 (Applied Precision personalDV).

Assessment of gross aneuploidy and supernumerary centrosomes resulting from unperturbed mitosis in primary cells from patients with FA

We wanted to determine whether cells lacking a functioning FA pathway would develop gross aneuploidy or acquire supernumerary centrosomes when mitosis was allowed to occur in the absence of chemical perturbation. Thus, fibroblasts from twelve FA patients—each with mutations in a different FA gene—were cultured in the absence of spindle poisons, fixed, and stained. DNA was labeled with Hoechst 33342, and endogenous pericentrin (a known centrosomal protein) was labeled via immunostaining. Images were acquired by deconvolution microscopy, and cells were manually quantified. Compared with control fibroblasts, primary fibroblasts from FA patients contained structural nuclear abnormalities (multinuclei and micronuclei) and abnormally high numbers of centrosomes.

a



Hoechst
DNA stain

Pericentrin (centrosome)
immunofluorescence
staining

Microscopy
images
captured

Primary fibroblasts isolated
from FA patients via skin biopsy
(12 complementation groups)

Quantification of centrosomes and structural nuclear abnormalities
in microscopy images

Normal cell division:

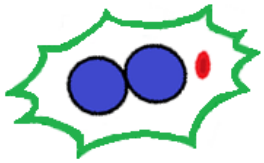
1 centrosome

2 centrosomes

Abnormal cell division:

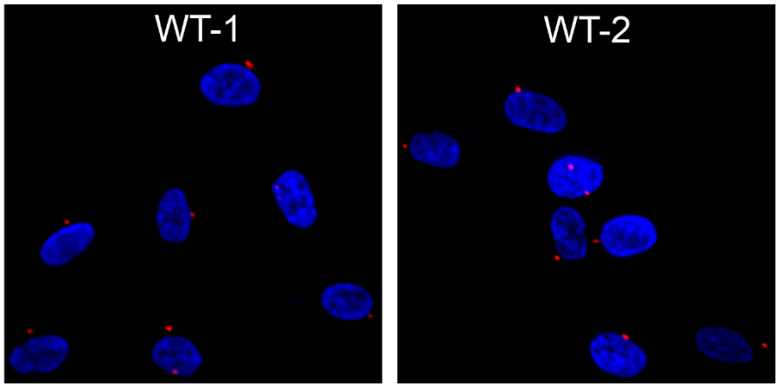
Supernumerary centrosomes

Micronucleus

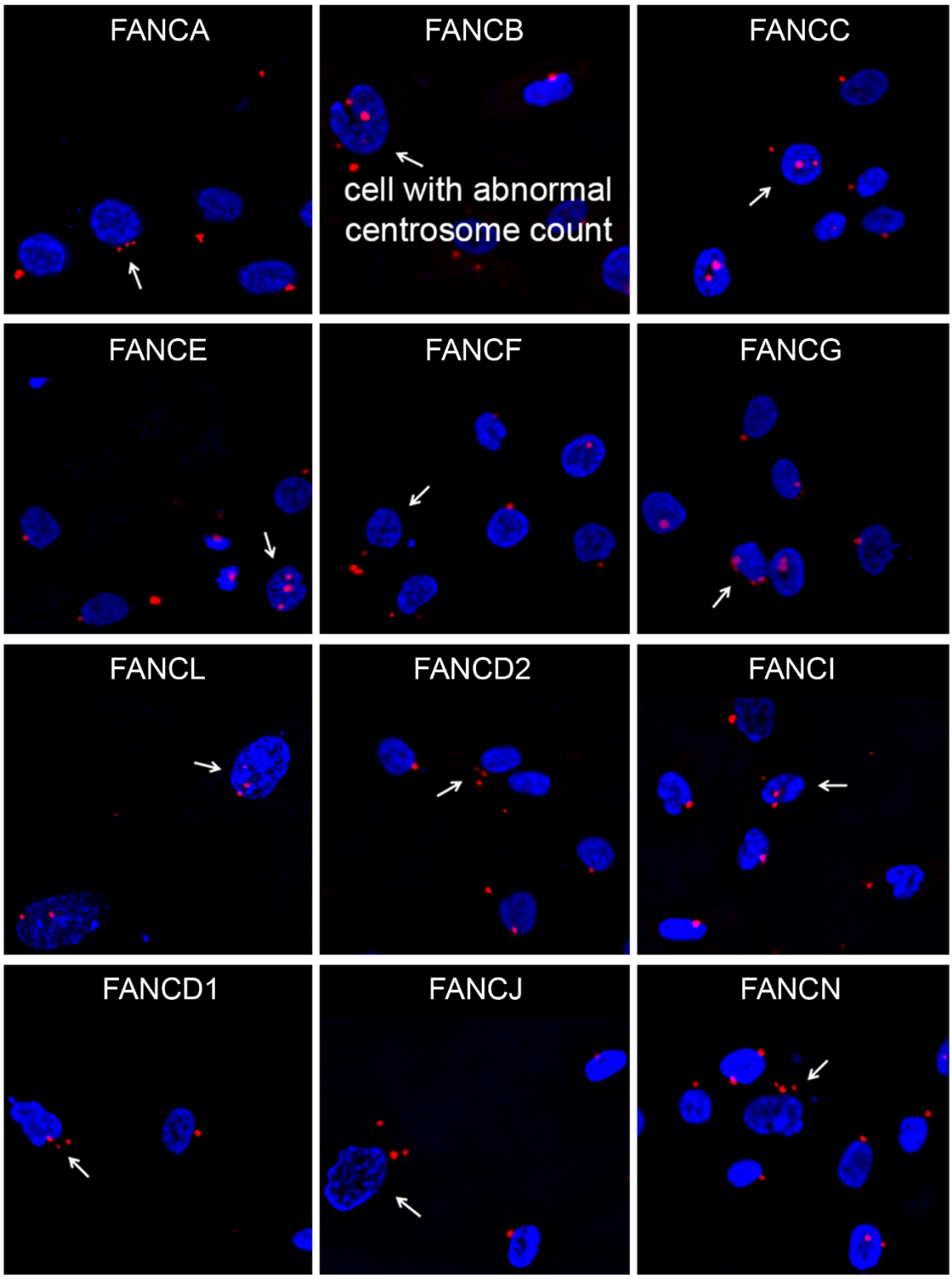


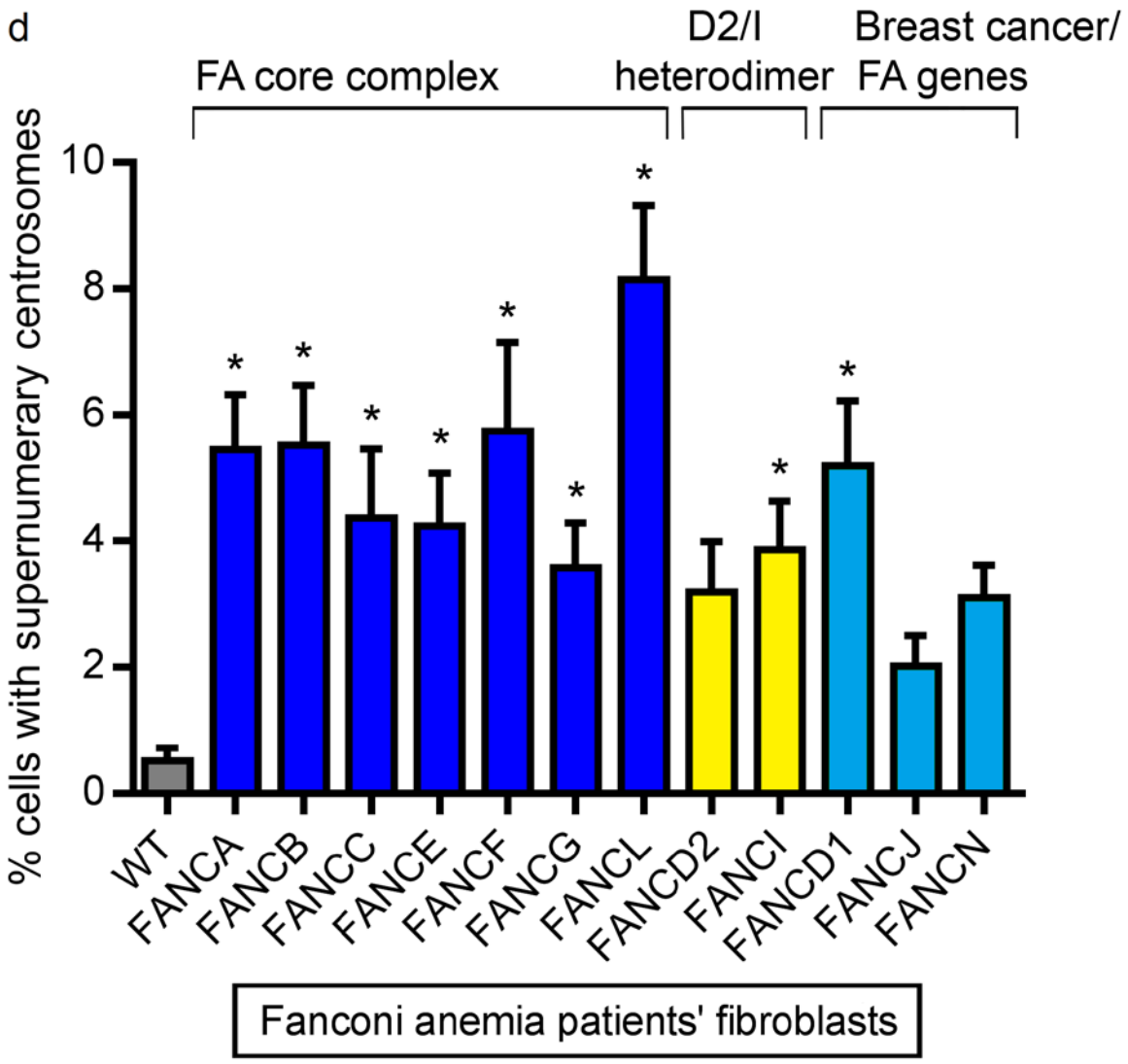
Multinucleation

b

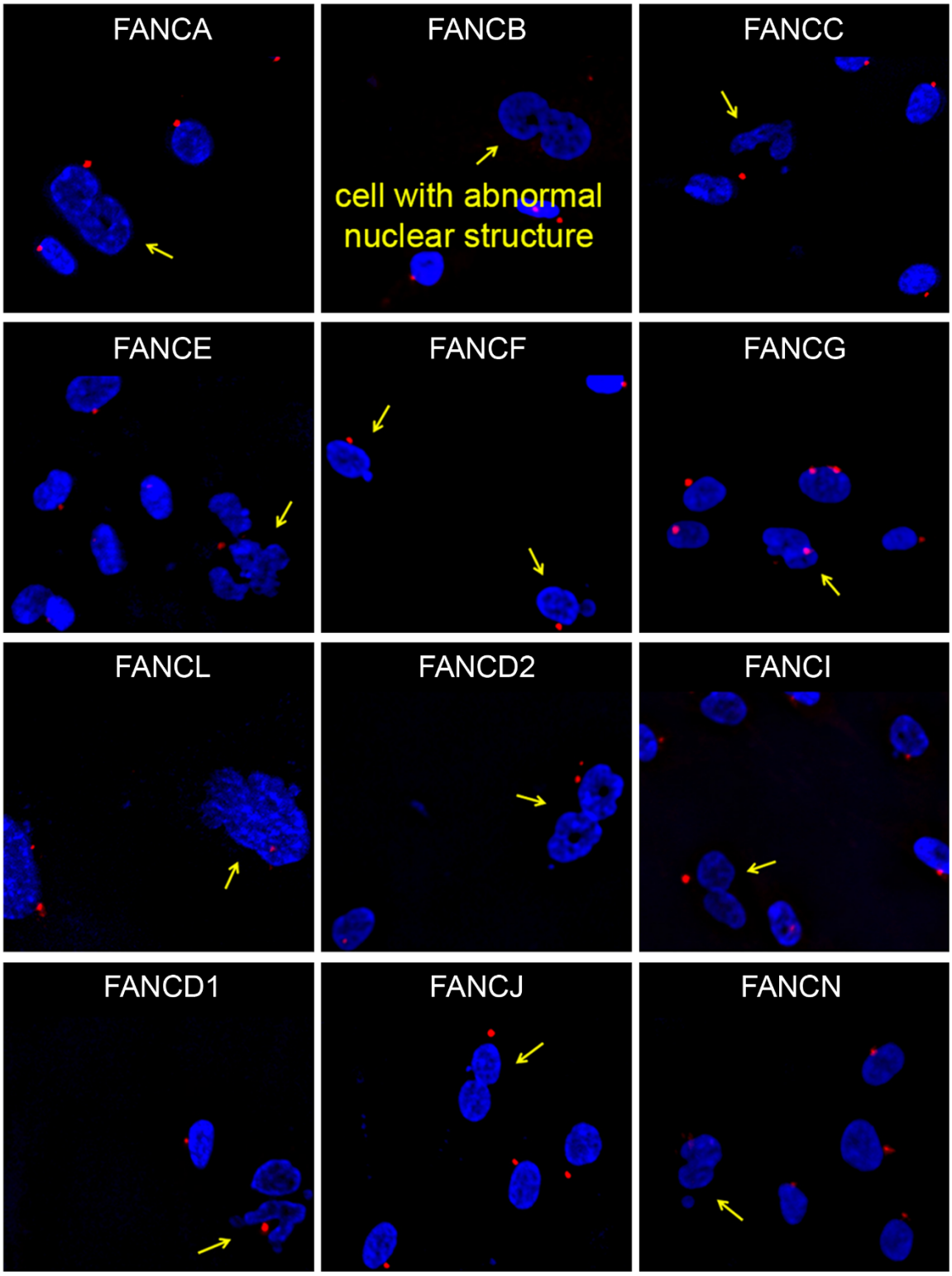


c


FANCA
FANCB
cell with abnormal centrosome count
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1
FANCJ
FANCN


d
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
% cells with supernumerary centrosomes
0
2
4
6
8
10
*
WT
FANCA
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1
FANCJ
FANCN
Fanconi anemia patients' fibroblasts

e


FANCA
FANCB
cell with abnormal nuclear structure
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1
FANCJ
FANCN

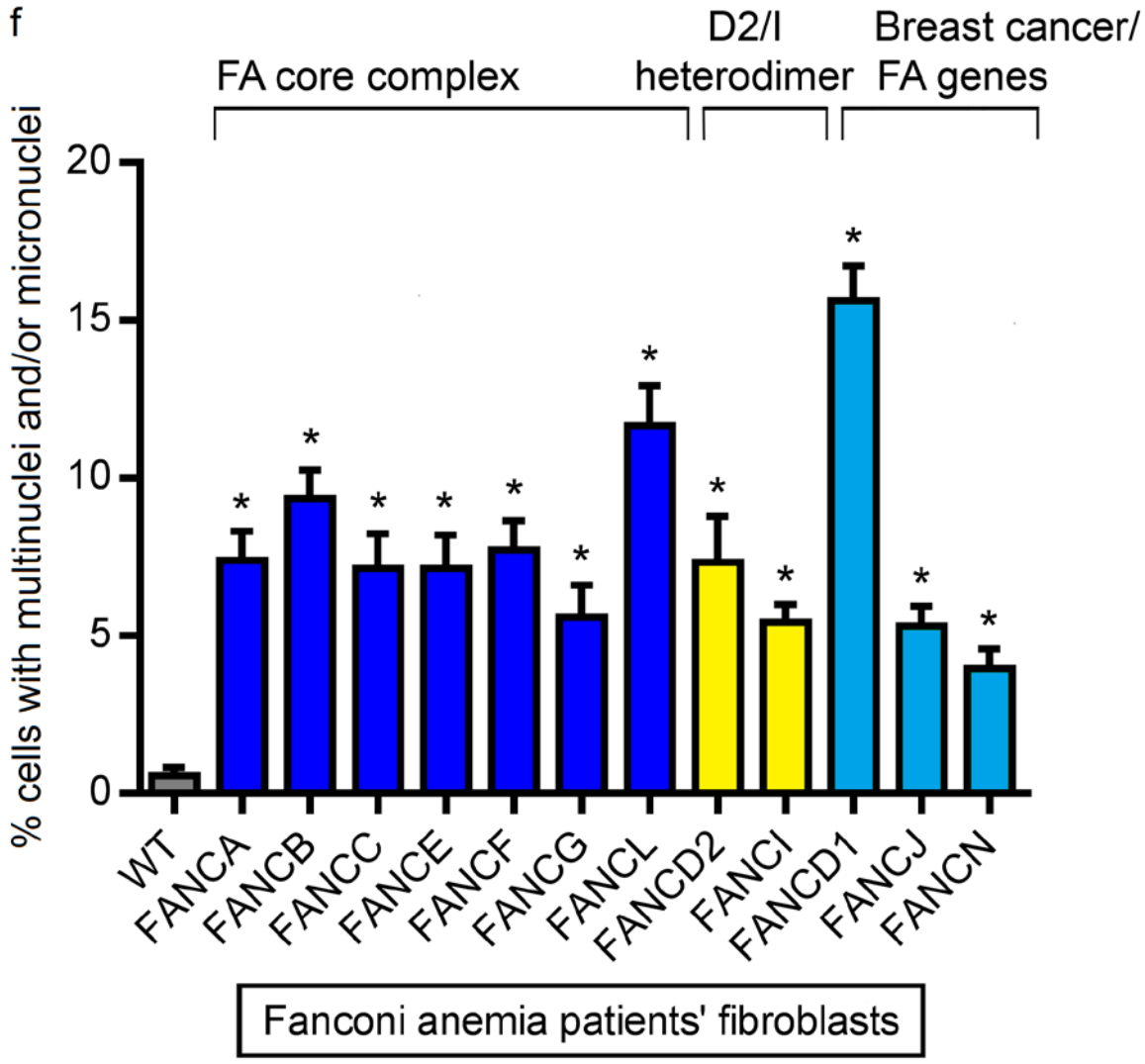
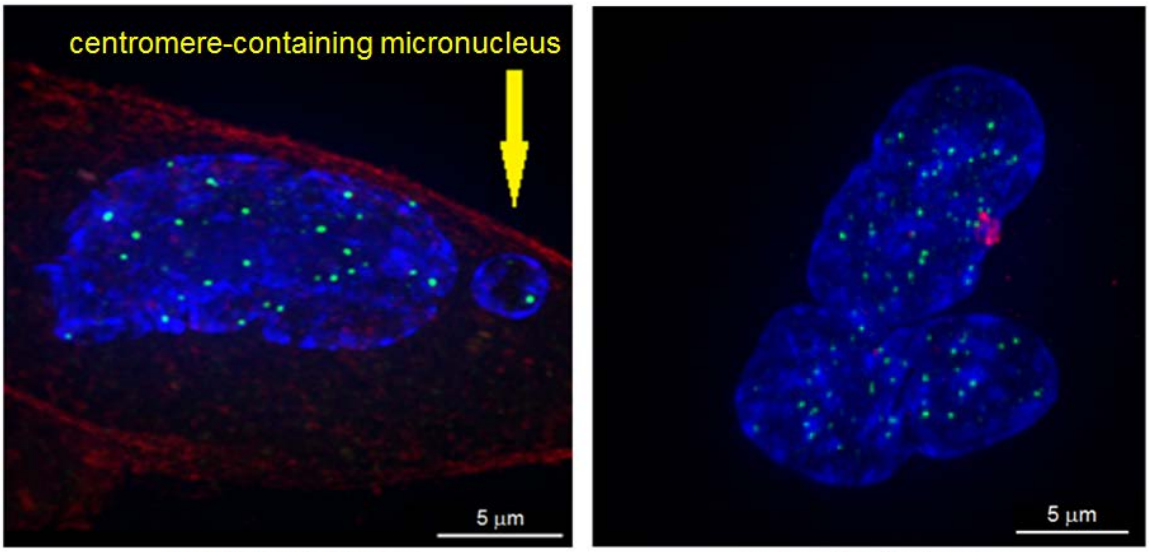

f
FA core complex
D2/I heterodimer
Breast cancer/ FA genes
% cells with multinuclei and/or micronuclei
20
15
10
5
0
*
WT
FANCA
FANCB
FANCC
FANCE
FANCF
FANCG
FANCL
FANCD2
FANCI
FANCD1
FANCJ
FANCN
Fanconi anemia patients' fibroblasts

Figure 4-2. Analysis of FA patient fibroblasts reveals supernumerary centrosomes and gross aneuploidy as a result of unperturbed mitosis. Abnormal centrosome counts and spontaneous micronucleation and multinucleation were detected in fibroblasts from patients with FA. **a)** Schematic of experimental design. **b)** As demonstrated by immunostaining of endogenous pericentrin (red), fibroblasts obtained from healthy controls (WT-1 and WT-2) contain one to two centrosomes per cell. Original magnification is ×200 (Applied Precision personalDV). **c)** Fibroblasts isolated from patients with FA contain supernumerary centrosomes (white arrows). Original magnification is ×200 (Applied Precision personalDV). **d)** Compared with healthy control fibroblasts, FA patient fibroblasts contain an increased percentage of cells with supernumerary centrosomes. Asterisks indicate $P < 0.05$ compared with the average of two healthy control fibroblast lines (1-way ANOVA with post-hoc Bonferroni's correction), $n = 30$ microscopic fields per fibroblast cell line, and all bars represent mean values ± SEM. **e)** Fibroblasts from patients with FA have abnormal nuclear structures and undergo spontaneous micronucleation and multinucleation (yellow arrows). Original magnification is ×200 (Applied Precision personalDV). **f)** Compared with healthy control fibroblasts, FA patient fibroblasts contain an increased percentage of cells with abnormal nuclei. Asterisks indicate $P < 0.05$ compared with the average of two healthy control fibroblast lines (1-way ANOVA with post-hoc Bonferroni's correction), $n = 30$ microscopic fields per fibroblast cell line, and all bars represent mean values ± SEM.

Detection of centromeres in micronuclei and multinuclei in primary cells from an FA patient of the FANCA subtype

Our study has established that the FA signaling network is essential for the mitotic SAC and that micronuclei form as a result of unperturbed mitosis in primary FA pathway-deficient cells, and previous studies have established an essential role for the FA signaling network in DNA damage repair. The presence of centromeres in micronuclei would strongly suggest the generation of micronuclei through defective chromosome segregation rather than DNA breakage. Thus, we wanted to determine whether some or all of the micronuclei and multinuclei in primary FA patient fibroblasts contain centromeres. Primary fibroblasts from a FANCA patient were cultured in the absence of spindle poisons, fixed, and stained. Endogenous CENPA (a known centromere protein) and endogenous pericentrin (a known centrosomal protein) were labeled via immunostaining, and DNA and actin respectively were labeled with Hoechst 33342 and AlexaFluor594-conjugated phalloidin. Images of primary FANCA patient-derived fibroblasts with structural nuclear abnormalities were acquired by deconvolution microscopy, and cells were manually quantified. Kinetochores were detected in most, but not all, of the micronuclei and multinuclei resulting from unperturbed mitosis in primary fibroblasts from an FA patient of the FANCA subtype. This finding indicates that both chromosome mis-segregation and DNA breaks can result in aneuploidy in primary FANCA-deficient cells. We conclude that chromosome mis-segregation due to weakened SAC activity is a major mechanism leading to the development of aneuploidy in FANCA-deficient cells.

a



green=kinetochores blue=DNA red=actin

green=kinetochores blue=DNA red=centrosome

b

Type of nuclear abnormality	# of cells	% of cells
Bi/multinucleated cells	6/25	24%
Cells with micronuclei	22/25	88%

c

	# of abnormal nuclei	% of abnormal nuclei
Abnormal nuclei with centromeres	29/42	69%

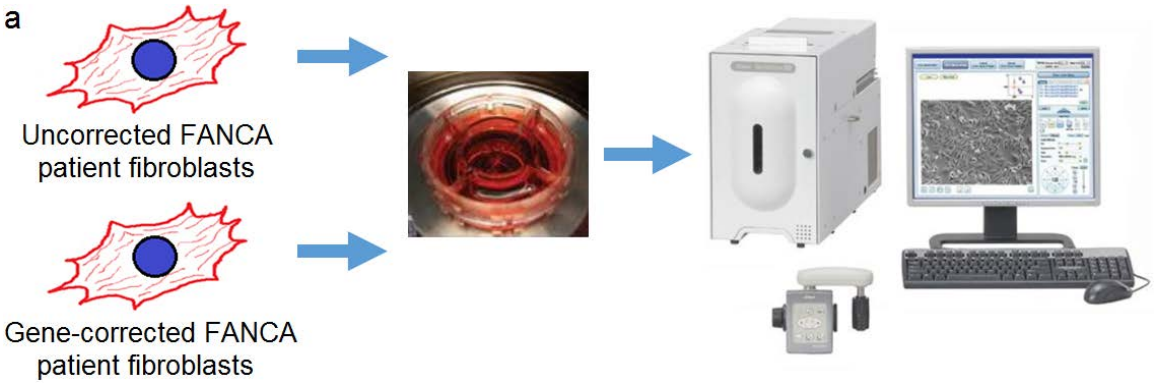
Figure 4-3. Immunostaining of CENPA in FANCA patient-derived fibroblasts reveals the presence of centromeres within micronuclei and multinuclei. Centromeres were labeled via immunostaining of endogenous CENPA in primary fibroblasts derived from an FA patient of the FANCA subtype. Twenty-five primary FANCA-deficient fibroblasts with structural nuclear abnormalities were imaged and manually quantified, and a total of forty-two abnormal nuclei were observed. **a)** In the image on the left, a micronucleus within a primary FANCA patient fibroblast contains a single centromere, suggesting that this micronucleus contains a single sister chromatid which mis-segregated during anaphase. The yellow arrow indicates the centromere-positive micronucleus. Centromeres are green, DNA is blue, and actin is red. In the image on the right, three nuclei in a multinucleated primary FANCA patient fibroblast contain centromeres. Centromeres are green, DNA is blue, and the centrosome is red. Representative images are shown, scale bars represent 5 um, and the original magnification is ×1,000 (Applied Precision personalDV). **b)** Table indicating the proportion of the quantified cells with each type of structural nuclear abnormality. **c)** Table indicating the proportion of abnormal nuclei which contain centromeres.

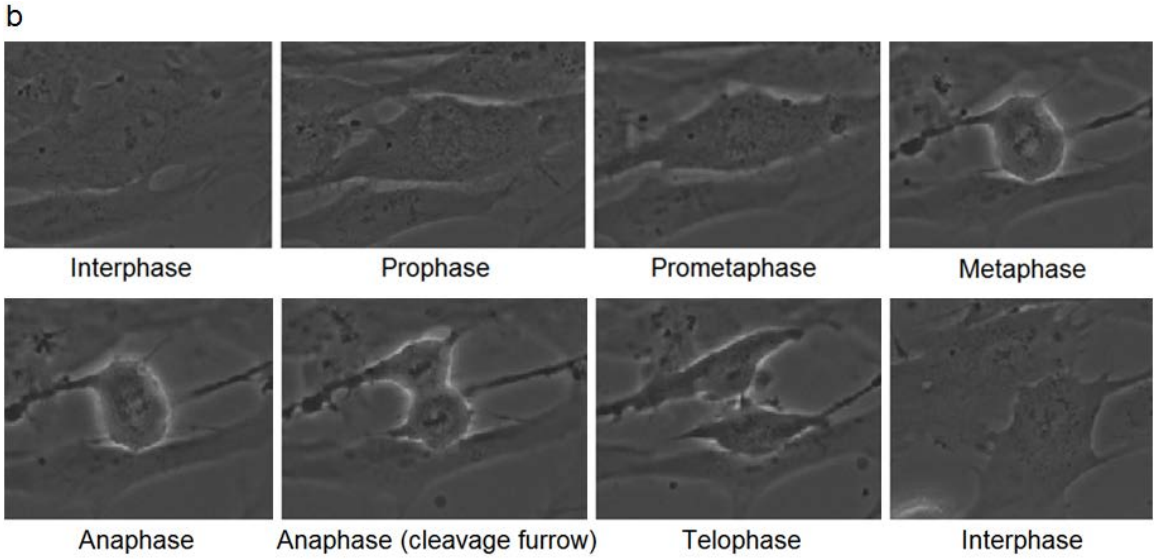
Systematic assessment of unperturbed mitosis in primary FANCA patient fibroblasts utilizing video microscopy

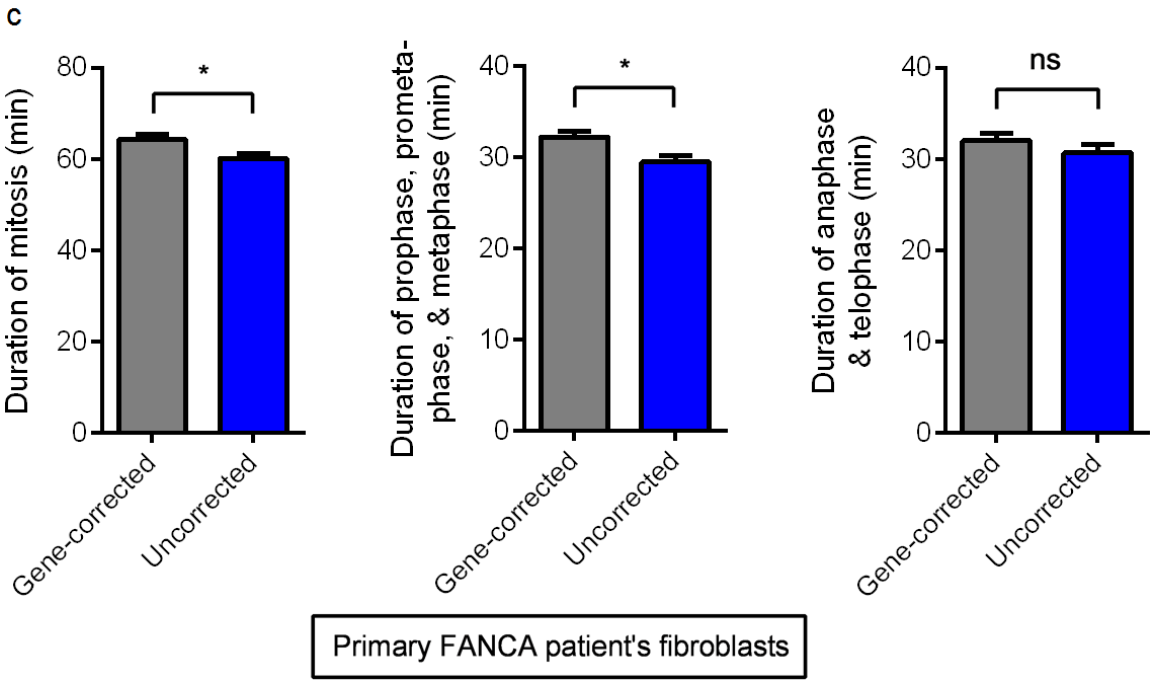
To quantify the duration of individual phases of mitosis and determine whether notable phenotypic defects occur in mitosis and/or cytokinesis in FA pathway-deficient cells, time-lapse microscopy of unperturbed mitosis was performed in uncorrected primary FANCA patient fibroblasts and isogenic gene-corrected control fibroblasts. Since weakened SAC activity leads to premature initiation of anaphase in the presence of lingering unattached kinetochores, we anticipated that dividing cells would progress through the early mitotic phases and enter anaphase more quickly in FANCA-deficient fibroblasts, compared with gene-corrected control fibroblasts. Live-cell video microscopy of FANCA patient-derived fibroblasts revealed accelerated progression through the early mitotic phases in uncorrected FANCA-deficient fibroblasts compared with gene-corrected control fibroblasts. Specifically, prophase and metaphase were each significantly shorter in duration in uncorrected FANCA-deficient fibroblasts than in gene-corrected control fibroblasts. The finding of accelerated early mitosis in FANCA-deficient fibroblasts is consistent with a role for FANCA in the activity of the mitotic SAC.

Additionally, analysis of cell division in FANCA-deficient fibroblasts via live-cell video microscopy revealed multiple novel phenotypes indicating defects in mitosis and cytokinesis. The observed phenotypes in primary FANCA-deficient fibroblasts include the following: 1) the absence of a clear metaphase plate, suggesting a defect in chromosome congression; 2) rotation of the dividing cell

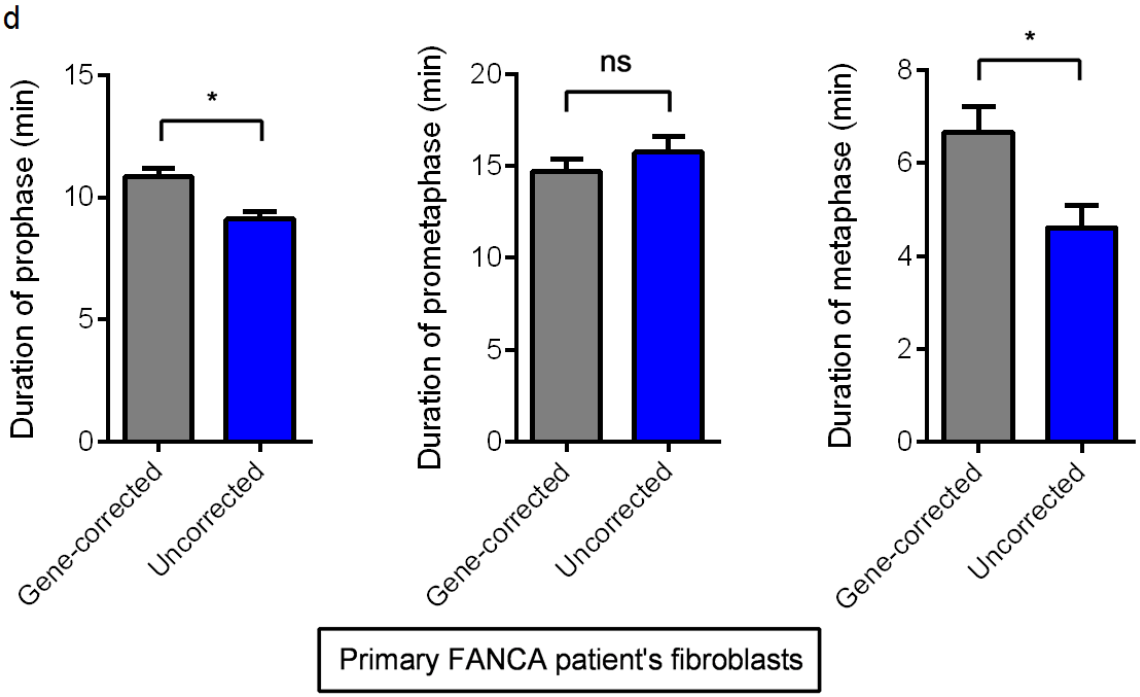
such that one newly forming daughter cell is vertically offset from the other during anaphase, indicating a defect in spindle orientation; 3) delayed nuclear envelope (NE) breakdown during prophase and asynchronous NE re-assembly during telophase, suggesting a defect in the regulation of the NE protein lamin; and 4) massive formation of vesicles at the cell surface during cytokinesis, suggesting a defect in the endocytic pathways which are essential for the addition of new membrane to the cleavage furrow during cytokinesis. The observation of spindle mis-orientation in FANCA-deficient fibroblasts has been validated in an experiment utilizing deconvolution microscopy to quantify the angle of abnormal spindle rotation in metaphase cells. This validation experiment is summarized in the next section and shown in Figure 4-5. Validation experiments for each of the other phenotypes are proposed in the Future Directions.

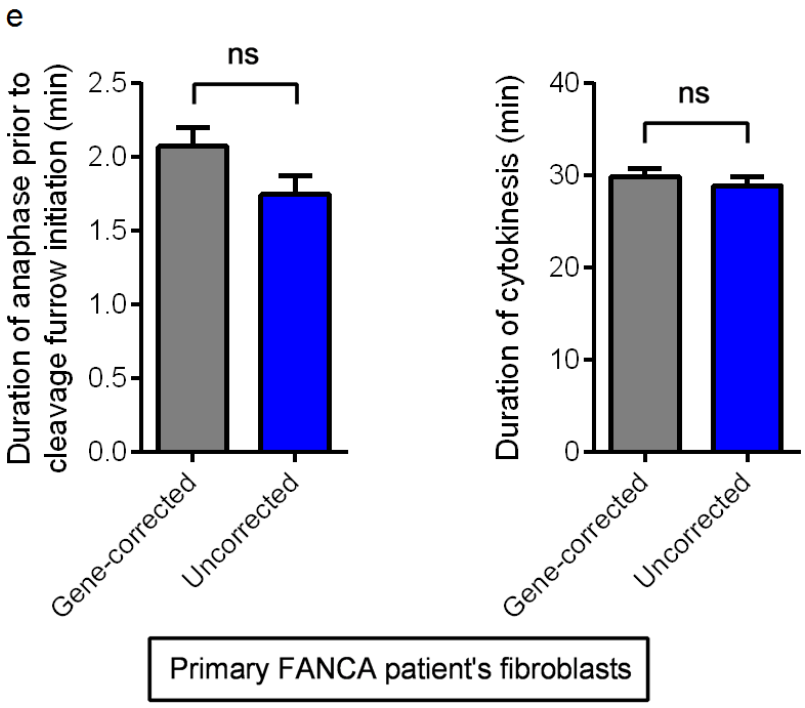


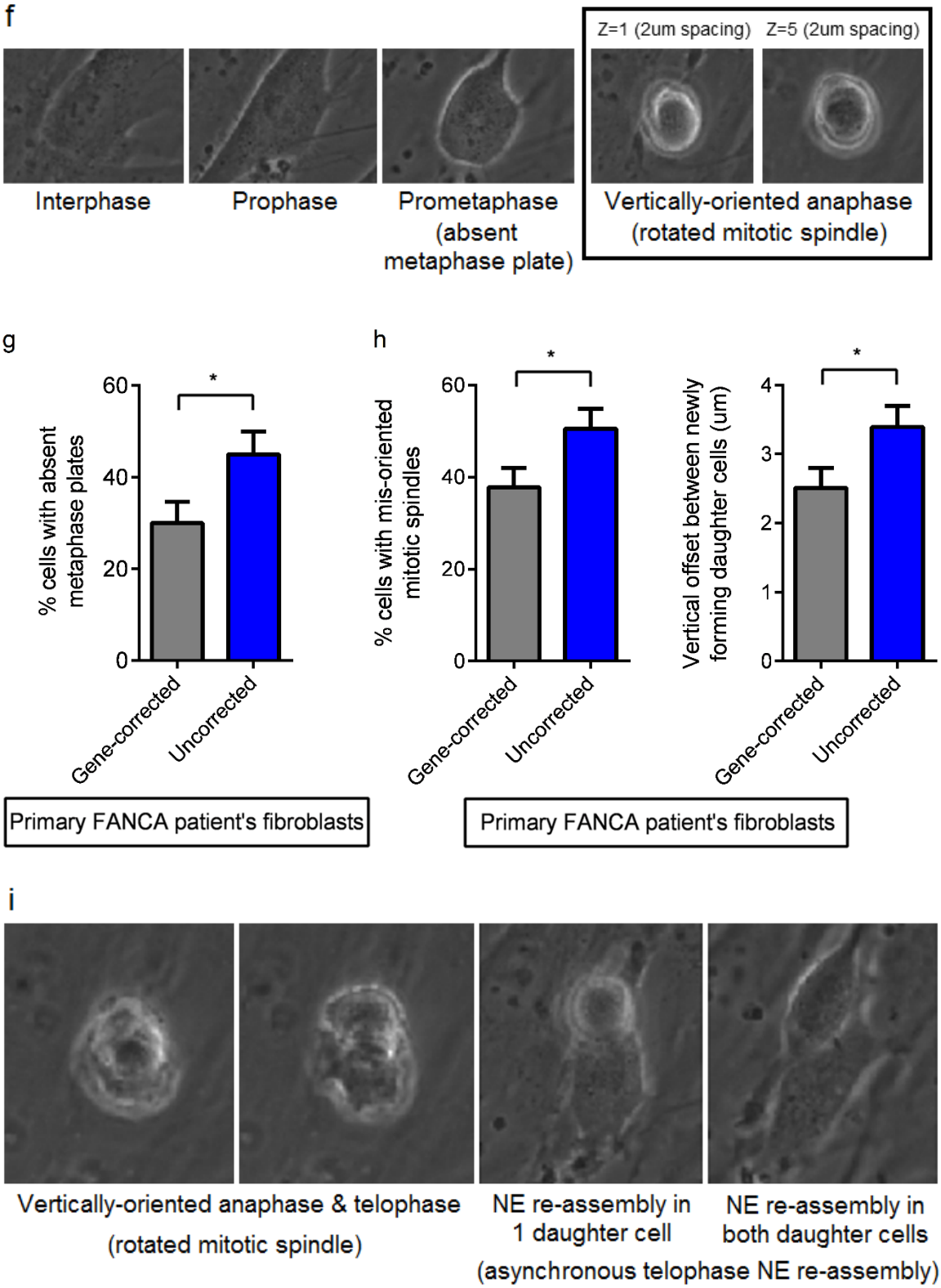


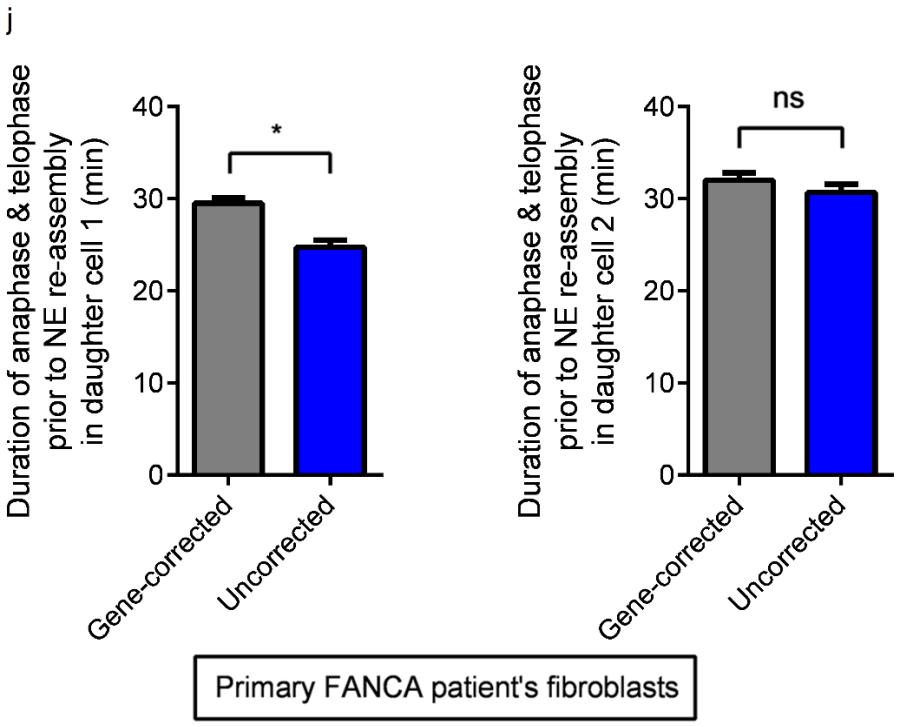


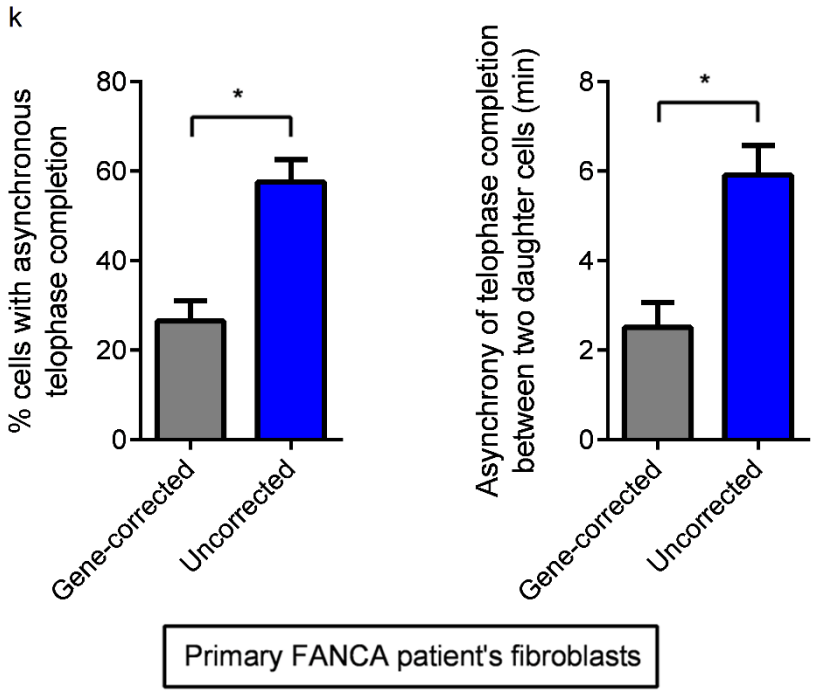
Primary FANCA patient's fibroblasts


d
Duration of prophase (min)
15
10
5
0
*
Gene-corrected
Uncorrected
Duration of prometaphase (min)
20
15
10
5
0
ns
Gene-corrected
Uncorrected
Duration of metaphase (min)
8
6
4
2
0
*
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts

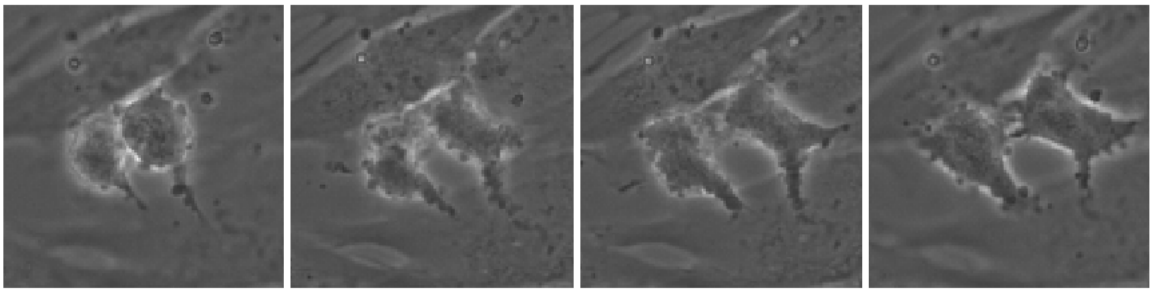

e
Duration of anaphase prior to cleavage furrow initiation (min)
2.5
2.0
1.5
1.0
0.5
0.0
ns
Gene-corrected
Uncorrected
Duration of cytokinesis (min)
40
30
20
10
0
ns
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts


f
Z=1 (2um spacing)
Z=5 (2um spacing)
Interphase
Prophase
Prometaphase (absent metaphase plate)
Vertically-oriented anaphase (rotated mitotic spindle)
g
% cells with absent metaphase plates
60
40
20
0
*
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts
h
% cells with mis-oriented mitotic spindles
60
40
20
0
*
Gene-corrected
Uncorrected
Vertical offset between newly forming daughter cells (um)
4
3
2
1
0
*
Gene-corrected
Uncorrected
Primary FANCA patient's fibroblasts
i
Vertically-oriented anaphase & telophase
(rotated mitotic spindle)
NE re-assembly in 1 daughter cell
NE re-assembly in both daughter cells
(asynchronous telophase NE re-assembly)


j
Duration of anaphase & telophase prior to NE re-assembly in daughter cell 1 (min)
40
30
20
10
0
*
Gene-corrected
Uncorrected
Duration of anaphase & telophase prior to NE re-assembly in daughter cell 2 (min)
ns
Primary FANCA patient's fibroblasts

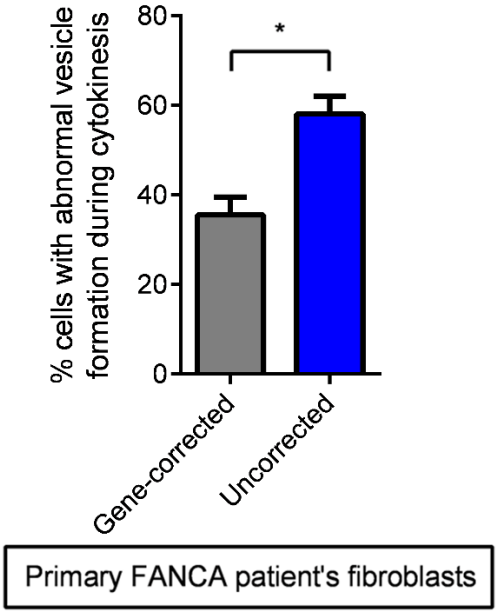

k
% cells with asynchronous telophase completion
80
60
40
20
0
*
Gene-corrected
Uncorrected
Asynchrony of telophase completion between two daughter cells (min)
8
6
4
2
0
Primary FANCA patient's fibroblasts

l

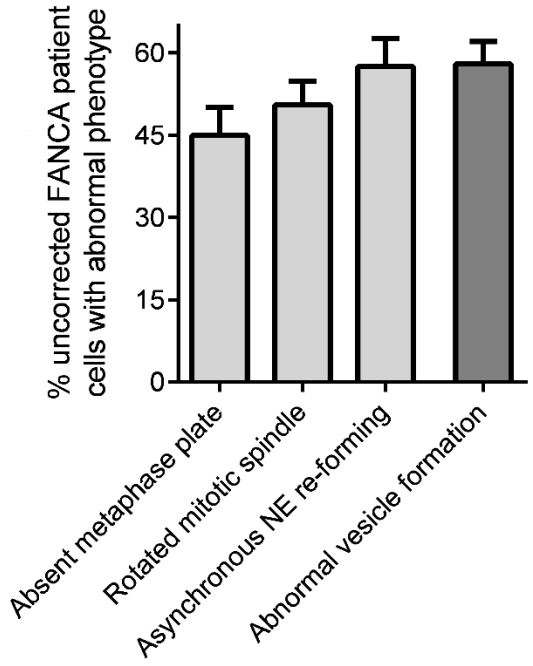


Abnormal vesicle formation during cytokinesis

m



n



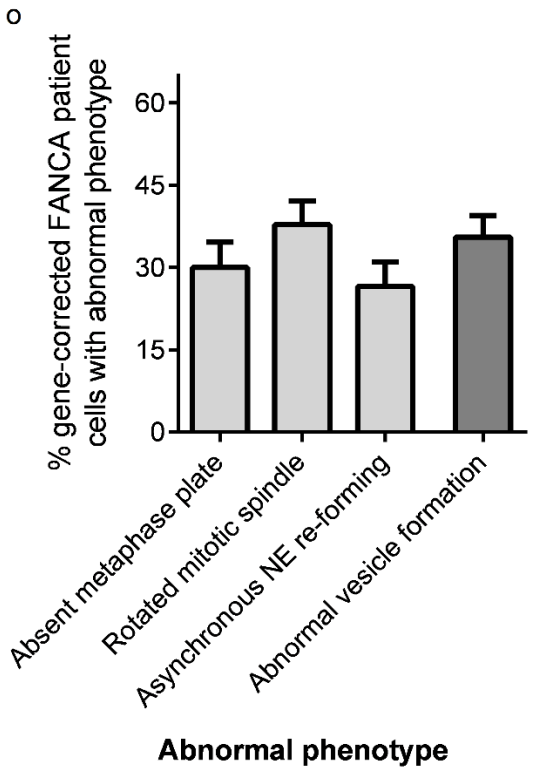
Pearson *r* values

	Absent metaphase plate	Rotated mitotic spindle	Asynchronous NE re-assembly	Abnormal vesicle formation
Absent metaphase plate		0.756	0.537	0.119
Rotated mitotic spindle	0.756		0.686	0.069
Asynchronous NE re-assembly	0.537	0.686		0.161
Abnormal vesicle formation	0.119	0.069	0.161	

***P* values**

	Absent metaphase plate	Rotated mitotic spindle	Asynchronous NE re-assembly	Abnormal vesicle formation
Absent metaphase plate		1.1E-19	9.8E-09	0.237
Rotated mitotic spindle	1.1E-19		5.0E-15	0.495
Asynchronous NE re-assembly	9.8E-09	5.0E-15		0.112
Abnormal vesicle formation	0.237	0.495	0.112	

o



Pearson *r* values

	Absent metaphase plate	Rotated mitotic spindle	Asynchronous NE re-assembly	Abnormal vesicle formation
Absent metaphase plate		0.428	0.403	0.185
Rotated mitotic spindle	0.428		0.611	0.163
Asynchronous NE re-assembly	0.403	0.611		0.057
Abnormal vesicle formation	0.185	0.163	0.057	

***P* values**

	Absent metaphase plate	Rotated mitotic spindle	Asynchronous NE re-assembly	Abnormal vesicle formation
Absent metaphase plate		1.1E-05	3.8E-05	0.066
Rotated mitotic spindle	1.1E-05		2.3E-11	0.109
Asynchronous NE re-assembly	3.8E-05	2.3E-11		0.574
Abnormal vesicle formation	0.066	0.109	0.574	

Figure 4-4. Video microscopy of unperturbed mitosis in primary FANCA patient fibroblasts reveals accelerated mitosis and novel phenotypic defects in mitosis and cytokinesis. Live-cell video microscopy of primary FANCA patient-derived fibroblasts was performed in the absence of spindle poisons to quantify the duration of mitotic phases and visualize defects in mitosis and cytokinesis. In all panels, *n* = 100 cells per genotype, original magnification is ×200 (Nikon BioStation IM-Q), and all bars represent mean values ± SEM. **a)** Schematic of experimental design. **b)** Representative images of a fibroblast progressing through each phase of mitosis. **c)** Compared with gene-corrected control fibroblasts, uncorrected FANCA-deficient fibroblasts exhibit decreased duration of mitosis (P = 0.0091) and decreased duration of early mitosis (prophase, prometaphase, and metaphase) (P = 0.0019). No difference was observed in the duration of late mitosis (anaphase and telophase) (P = 0.2642). **d)** Compared with control fibroblasts, FANCA-deficient fibroblasts exhibit accelerated prophase (P = 0.0002) and metaphase (P = 0.0050), but not prometaphase (P = 0.3256). **e)** FANCA-deficient fibroblasts exhibit no discernable difference in the timing of cytokinesis when compared with control fibroblasts. The timing of the onset of cytokinesis (duration of anaphase prior to cleavage furrow initiation) is not significantly altered in FANCA-deficient fibroblasts (P = 0.0751), nor is the duration of cytokinesis from cleavage furrow initiation to the completion of NE re-assembly (P = 0.4240). **f)** Representative images of a FANCA-deficient fibroblast progressing through mitosis which exhibits an absent metaphase plate and rotated mitotic spindle. **g)** Compared with control fibroblasts, a greater percentage of FANCA-deficient fibroblasts lack a clearly defined metaphase plate (P = 0.0285). **h)** FANCA-deficient fibroblasts exhibit mis-oriented mitotic spindles, evidenced by rotation of the dividing cell out of the normal horizontal plane, so that one newly forming daughter cell is vertically offset from the other during anaphase. Compared with control fibroblasts, a greater percentage of FANCA-deficient fibroblasts exhibit spindle mis-orientation (graph on left; P = 0.0384), and the offset between the two daughter cells is greater in FANCA-deficient fibroblasts (P = 0.0395). **i)** Representative images of a FANCA-deficient fibroblast progressing through mitosis which exhibits asynchronous NE re-assembly during telophase. **j)** Compared with control fibroblasts, FANCA-deficient fibroblasts exhibit earlier nuclear envelope (NE) re-assembly in the first daughter cell (graph on left; P < 0.0001), but no difference in the duration of late mitotic phases prior to NE re-assembly in the second daughter cell (graph on right; P = 0.2642). This means that NE re-assembly occurs at different times for each daughter cell in uncorrected FANCA patient fibroblasts. **k)** FANCA-deficient fibroblasts exhibit asynchronous NE re-assembly between the two newly forming daughter cells during telophase. Compared with control fibroblasts, a greater percentage of FANCA-deficient fibroblasts exhibit asynchronous telophase NE re-assembly (graph on left; P < 0.0001) and a greater duration of asynchrony occurs in FANCA-deficient fibroblasts (graph on right; P = 0.0001). **l)** Representative images of a FANCA-deficient fibroblast progressing through cytokinesis which exhibits abnormal vesicle formation. **m)** A greater percentage of primary FANCA-

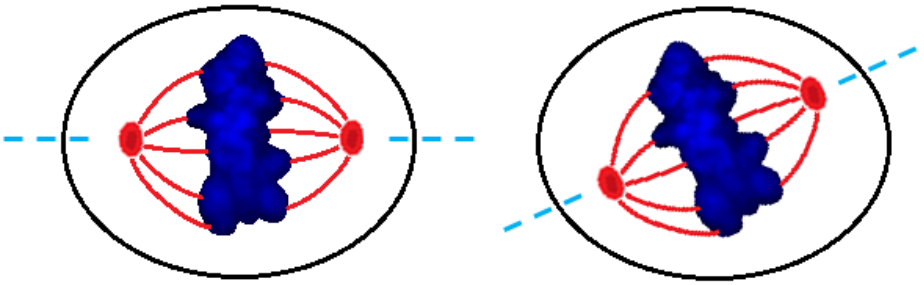
deficient fibroblasts exhibit apparent defects in endocytic vesicle formation during cytokinesis, compared with control fibroblasts (P = 0.0001). **n and o)** In both uncorrected and gene-corrected primary FANCA patient fibroblasts, the observed phenotypic defects are significantly correlated with one another (P < 0.05), except for abnormal vesicle formation during cytokinesis. In **n and o,** bars in the same color represent phenotypes which are correlated. Pearson r values range from 0 to 1 (0 = no correlation, 1 = complete correlation).

Quantification of spindle mis-orientation in primary fibroblasts from an FA patient of the FANCA subtype

In the previous experiment, live-cell video microscopy revealed abnormal orientation of the mitotic spindle in primary FANCA-deficient fibroblasts. During anaphase, chromosome segregation was rotated out of the normal horizontal plane and one newly forming daughter cell was vertically offset from the other in uncorrected FANCA patient fibroblasts, compared with gene-corrected control fibroblasts. Quantification of the vertical offset between the two newly forming daughter cells gave a crude estimation of spindle orientation.

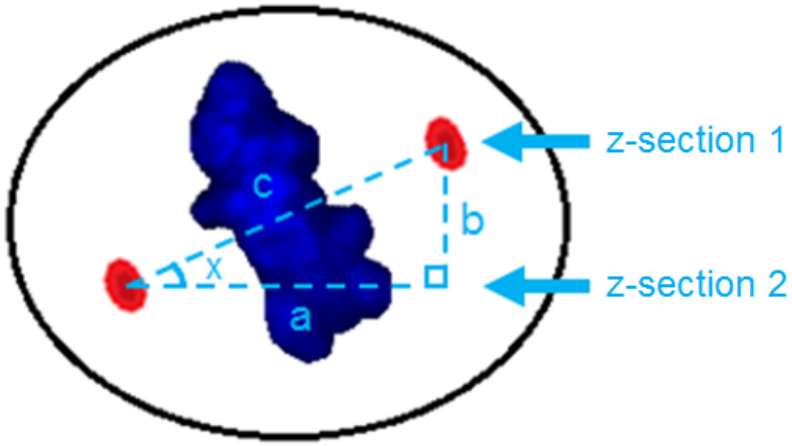
To validate the phenotype of spindle mis-orientation, we wanted to precisely quantify the angle of spindle orientation with respect to the horizontal plane in uncorrected and gene-corrected FANCA-deficient fibroblasts during metaphase. Thus, uncorrected and gene-corrected primary FANCA patient-derived fibroblasts were treated with the proteasome inhibitor MG132 for 4 hours. Since the initiation of anaphase is dependent on proteasomal degradation of the targets of APC/C, treatment with MG132 increases the number of metaphase cells by preventing anaphase entry. Immunostaining of pericentrin and CENPA (to label centrosomes and kinetochores respectively) and staining with Hoechst 33342 and fluorophore-conjugated phalloidin (to label DNA and actin respectively) were performed. Using basic trigonometry calculations, the angle of spindle orientation with respect to the horizontal plane was quantified based on the relative positioning of centrosomes. This experiment confirmed that the mitotic spindle is oriented abnormally in dividing FANCA-deficient fibroblasts.

a



Normal spindle orientation Abnormal spindle orientation

b

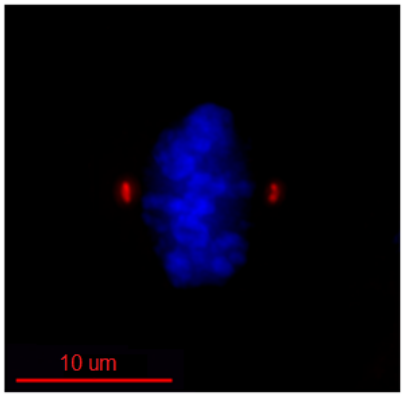


Calculation of spindle angle x

c

Gene-corrected FANCA patient fibroblast

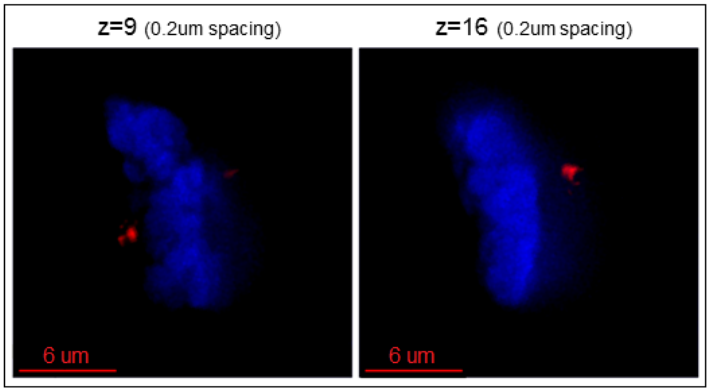
Metaphase



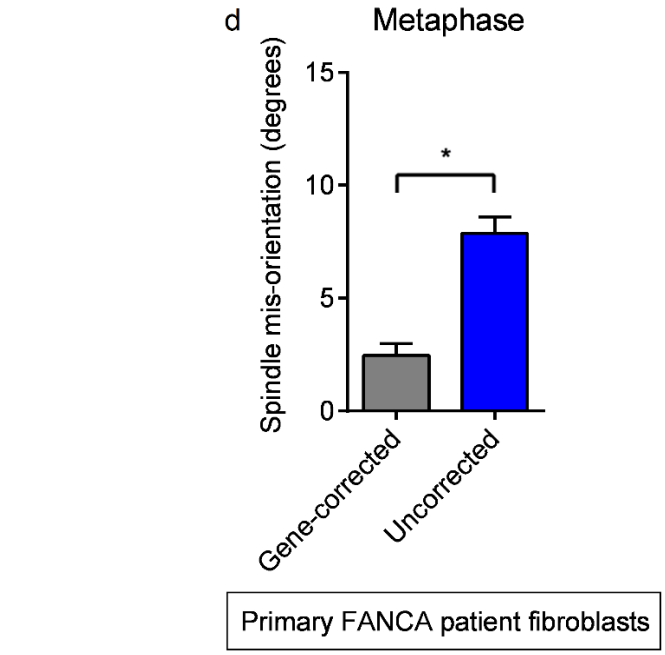
The mitotic spindle is oriented approx. **0°** from horizontal.

Uncorrected FANCA patient fibroblast

Metaphase



The mitotic spindle is oriented approx. **9°** from horizontal.


d
Metaphase
Spindle mis-orientation (degrees)
15
10
5
0
*
Gene-corrected
Uncorrected
Primary FANCA patient fibroblasts

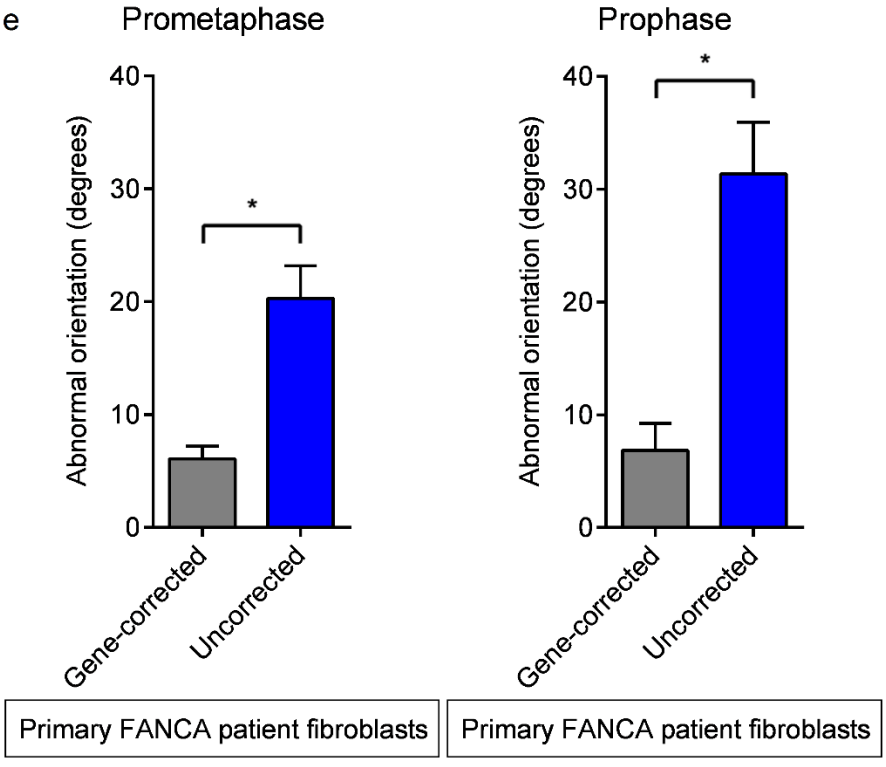

e
Prometaphase
Abnormal orientation (degrees)
40
30
20
10
0
*
Gene-corrected
Uncorrected
Primary FANCA patient fibroblasts
Prophase
Abnormal orientation (degrees)
40
30
20
10
0
*
Gene-corrected
Uncorrected
Primary FANCA patient fibroblasts

Figure 4-5. Quantification of spindle angle confirms that primary FANCA-deficient fibroblasts exhibit mis-orientation of the mitotic spindle.
Uncorrected and gene-corrected primary FANCA patient-derived fibroblasts were treated with the proteasome inhibitor MG132 for 4 hours to prevent anaphase entry. Immunofluorescence staining of endogenous pericentrin and CENPA was performed to label centrosomes and kinetochores respectively, and relative positioning of the centrosomes was used to quantify the angle of spindle orientation with respect to the horizontal plane. Total *n* = 91 to 111 cells per genotype pooled from three independent experiments, original magnification is ×600 (Applied Precision personalDV), and all bars represent mean values ± SEM. **a)** Schematic diagram of cells with normal and abnormal orientation of the mitotic spindle, relative to the horizontal plane. DNA is blue, and the centrosomes and spindle are red. **b)** Schematic diagram illustrating how trigonometry can be used to calculate the angle of spindle mis-orientation, relative to the horizontal plane. The sine of the angle x equals the height of the indicated right triangle, divided by the inter-centrosomal distance. The height of the triangle and the inter-centrosomal distance were determined using softWoRx Explorer software to analyze 3-d microscopic images comprised of multiple 2-d z-sections captured in parallel horizontal planes. **c)** Representative images of metaphase cells. **d)** Compared with gene-corrected control fibroblasts, uncorrected FANCA-deficient fibroblasts exhibit abnormal orientation of the mitotic spindle during metaphase ($P < 0.0001$). *n* = 50 to 68 metaphase cells per genotype. **e)** Compared with gene-corrected control fibroblasts, uncorrected FANCA-deficient fibroblasts are oriented abnormally during prometaphase ($P < 0.0001$) and during prophase ($P < 0.0001$). *n* = 21 prometaphase cells and 20 to 22 prophase cells per genotype.

CHAPTER FIVE

FA PROTEINS LOCALIZE TO THE MITOTIC APPARATUS

Introduction

Chapter three summarized our discovery that the FA signaling network plays an essential role in the regulation of the human mitotic SAC. Then, in chapter four, unperturbed mitosis was systemically examined in FA pathway-deficient cells. As described in the introduction, the kinetochore and the kinetochore-spindle fiber interface are the major sites of mitotic SAC regulation. Since we discovered that the FA signaling network is essential for the mitotic SAC and centrosome maintenance, we hypothesized that FA proteins localize to the mitotic apparatus, including the centrosomes, mitotic spindle, and kinetochores. We decided to systematically analyze the localization of the FA proteins in mitotic cells via immunofluorescence imaging studies, imaging studies utilizing ectopically expressed GFP-fused FA proteins, and studies utilizing biochemical fractionation followed by immunoblotting. Our key findings are summarized in the current chapter.

Results

FANCA localizes to the centrosome and spindle during mitosis

To determine whether FANCA localizes to centrosomes, kinetochores, and/or the mitotic spindle, immunofluorescence staining of endogenous FA proteins was performed in a HeLa cell line stably expressing GFP-fused γtubulin and GFP-fused CENPA. γtubulin is a centrosomal protein, and CENPA is a structural component of the inner kinetochore. Kinetochores are located on chromosomes at the centromere region. Centrosomes are larger, oval-shaped structures which are located at the spindle poles during mitosis. The mitotic spindle can be identified emanating from the centrosomes and, during metaphase, projecting from centrosomes to kinetochores. DNA staining was accomplished using Hoechst 33342. Localization was analyzed in immunostained cells by deconvolution microscopy. Mitotic cells of each phase were identified based on their nuclear morphology and centrosomal positioning. Localization of FANCA to centrosomes occurred in a cell cycle-dependent fashion throughout the mitotic phases, and localization of FANCA to the mitotic spindle was identified during the early phases of mitosis. Representative microscopy images are shown.

Endogenous FANCA localizes to the centrosomes and mitotic spindle

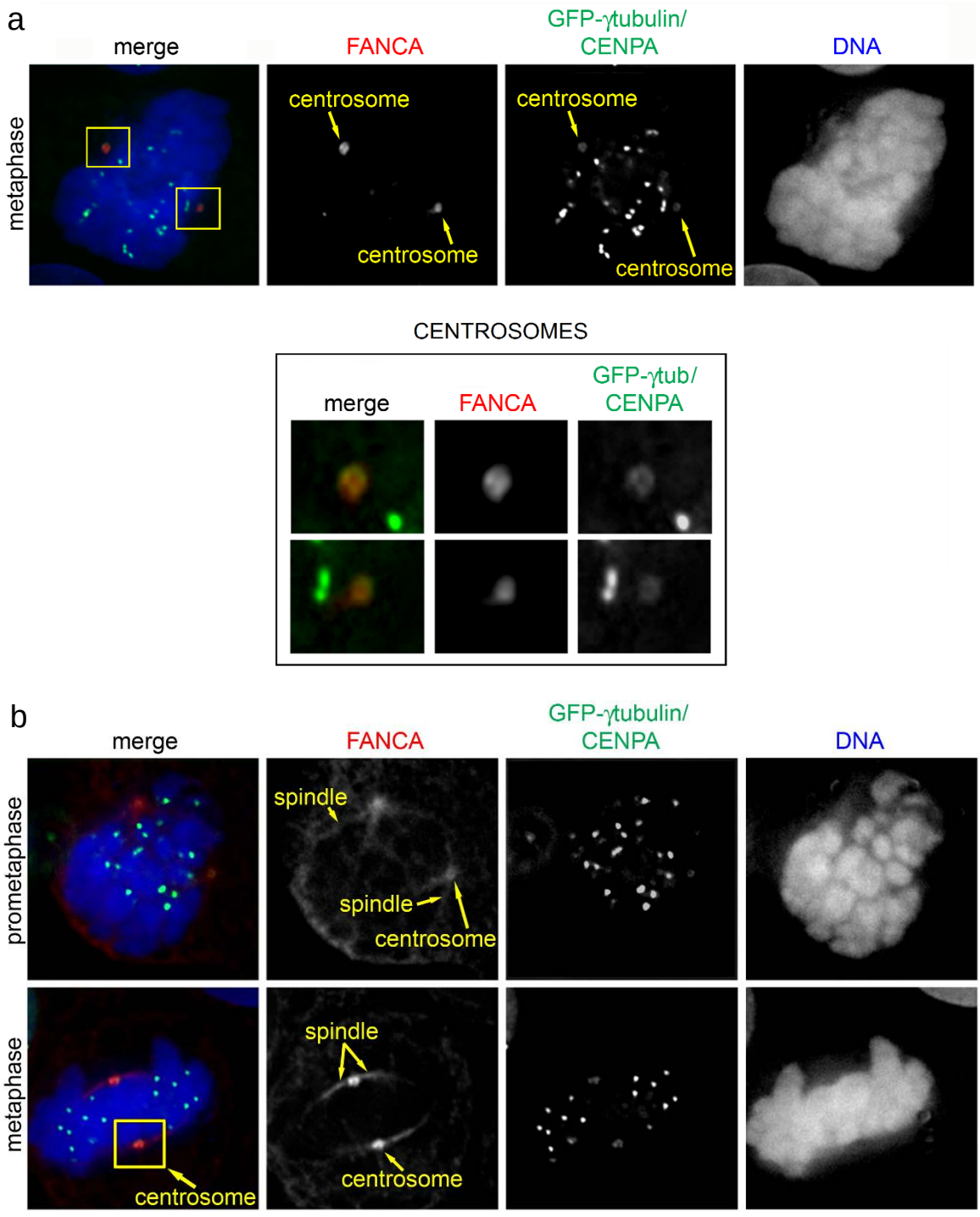


Figure 5-1. Deconvolution microscopy reveals that endogenous FANCA localizes to the centrosome and spindle during mitosis. a) Endogenous FANCA (red) co-localizes with GFP-γtubulin (green) at the centrosomes in a metaphase cell. Yellow arrows and boxes indicate centrosomes. The enlarged centrosomes below the main panel are the ones enclosed by yellow boxes in the main panel. Original magnification is ×1,000 (Applied Precision personalDV). **b)** Endogenous FANCA (red) localizes to the mitotic spindle during prophase and metaphase. Additionally, similar to **a**, endogenous FANCA co-localizes with GFP-γtubulin (green) at the centrosomes during prometaphase and metaphase. Yellow arrows and box indicate the mitotic spindle and centrosomes, as indicated. Original magnification is ×1,000 (Applied Precision personalDV).

Numerous FA proteins localize to the centrosome during mitosis

We wanted to determine whether FA proteins localize to centrosomes, kinetochores, and/or the mitotic spindle. Three approaches were utilized in experiments showing localization of FA proteins to the centrosome. Immunofluorescence staining of endogenous FA proteins was performed in a HeLa cell line stably expressing GFP-fused γtubulin and GFP-fused CENPA (γtubulin is a centrosomal protein, and CENPA is a structural component of the inner kinetochore), co-immunofluorescence staining of endogenous FA protein with αtubulin was performed in a HeLa cell line, and/or HeLa cells were transfected with constructs expressing GFP-fused FA protein. Centrosomes were identified in HeLa$^{\text{GFP-γtubulin/GFP-CENPA}}$ cells based on their large, oval-shaped morphology and their location at mitotic spindle poles. DNA staining was accomplished using Hoechst 33342. Localization was analyzed in immunostained cells by deconvolution microscopy. Mitotic cells of each phase were identified based on their nuclear morphology and centrosomal positioning. Localization of each FA protein to centrosomes occurred in a cell cycle-dependent fashion throughout the mitotic phases. Representative microscopy images are shown.

Endogenous FANCB localizes to the centrosome

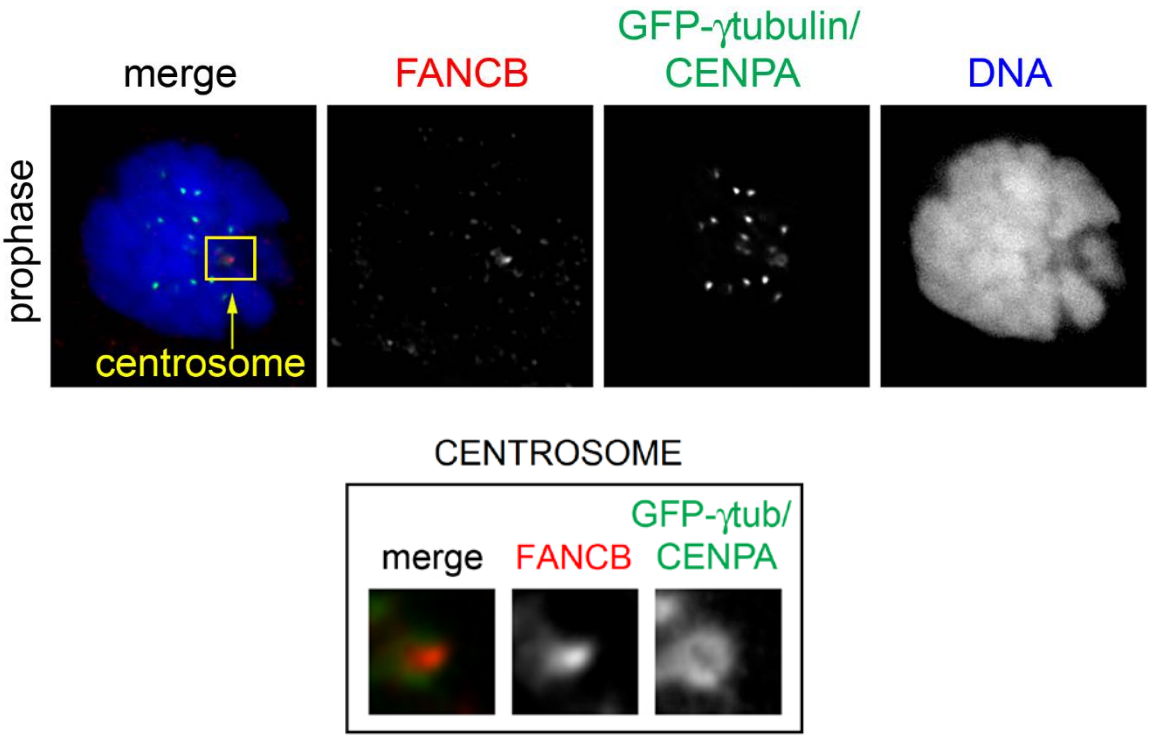


Figure 5-2. Deconvolution microscopy reveals that endogenous FANCB localizes to the centrosome during mitosis. Endogenous FANCB (red) co-localizes with GFP-γtubulin (green) at the centrosome in a prophase cell. The yellow arrow and box indicate a centrosome. The enlarged centrosome below the main panel is the one enclosed by a yellow box in the main panel. Original magnification is ×1,000 (Applied Precision personalDV).

Endogenous FANCE localizes to the centrosome

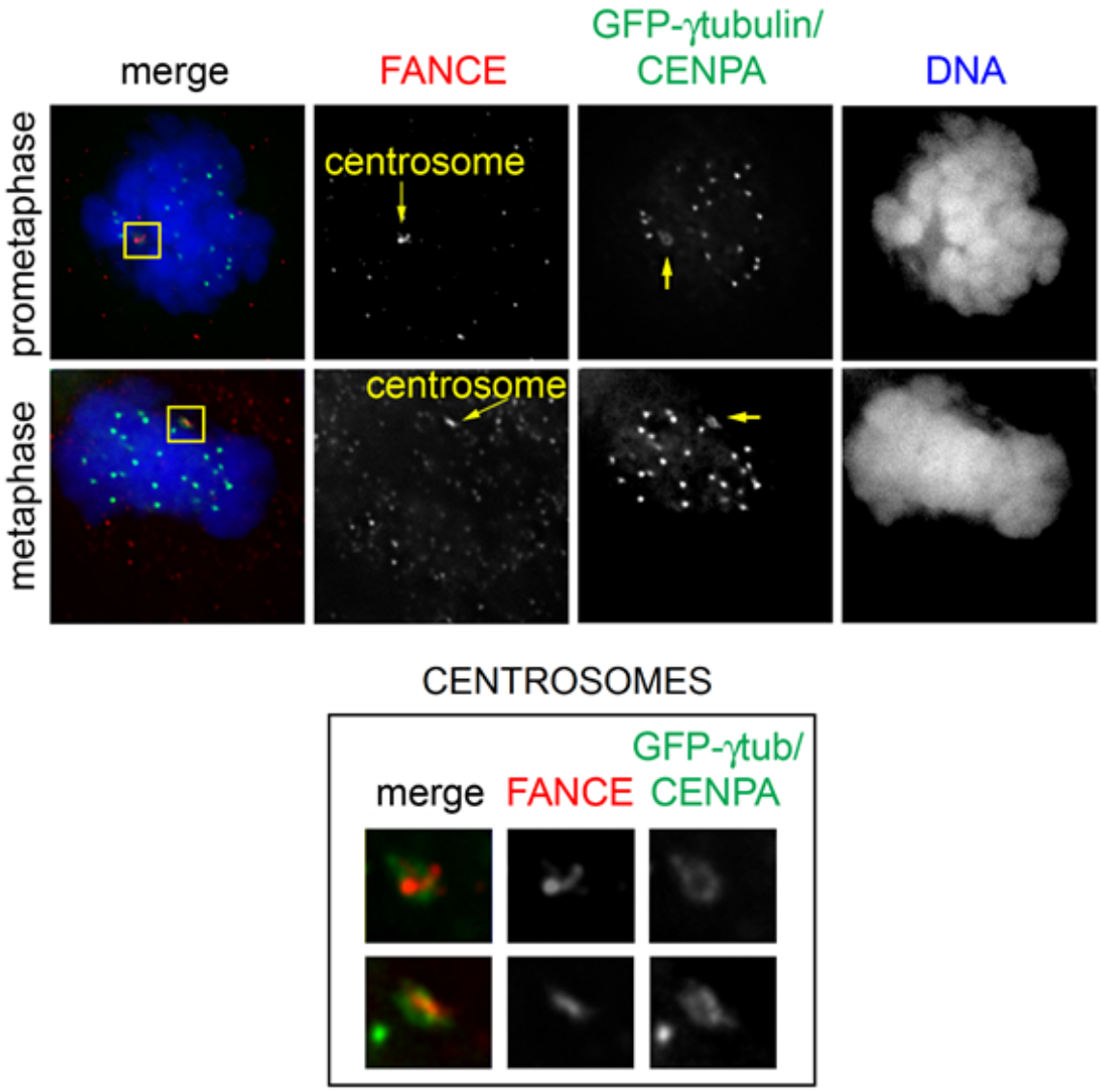


Figure 5-3. Deconvolution microscopy reveals that endogenous FANCE localizes to the centrosome during mitosis. Endogenous FANCE (red) co-localizes with GFP-γtubulin (green) at the centrosome in prometaphase and metaphase cells. Yellow arrows and boxes indicate centrosomes. The enlarged centrosomes below the main panel are the ones enclosed by yellow boxes in the main panel. Original magnification is ×1,000 (Applied Precision personalDV).

GFP-fused and endogenous FANCG localize to the centrosome

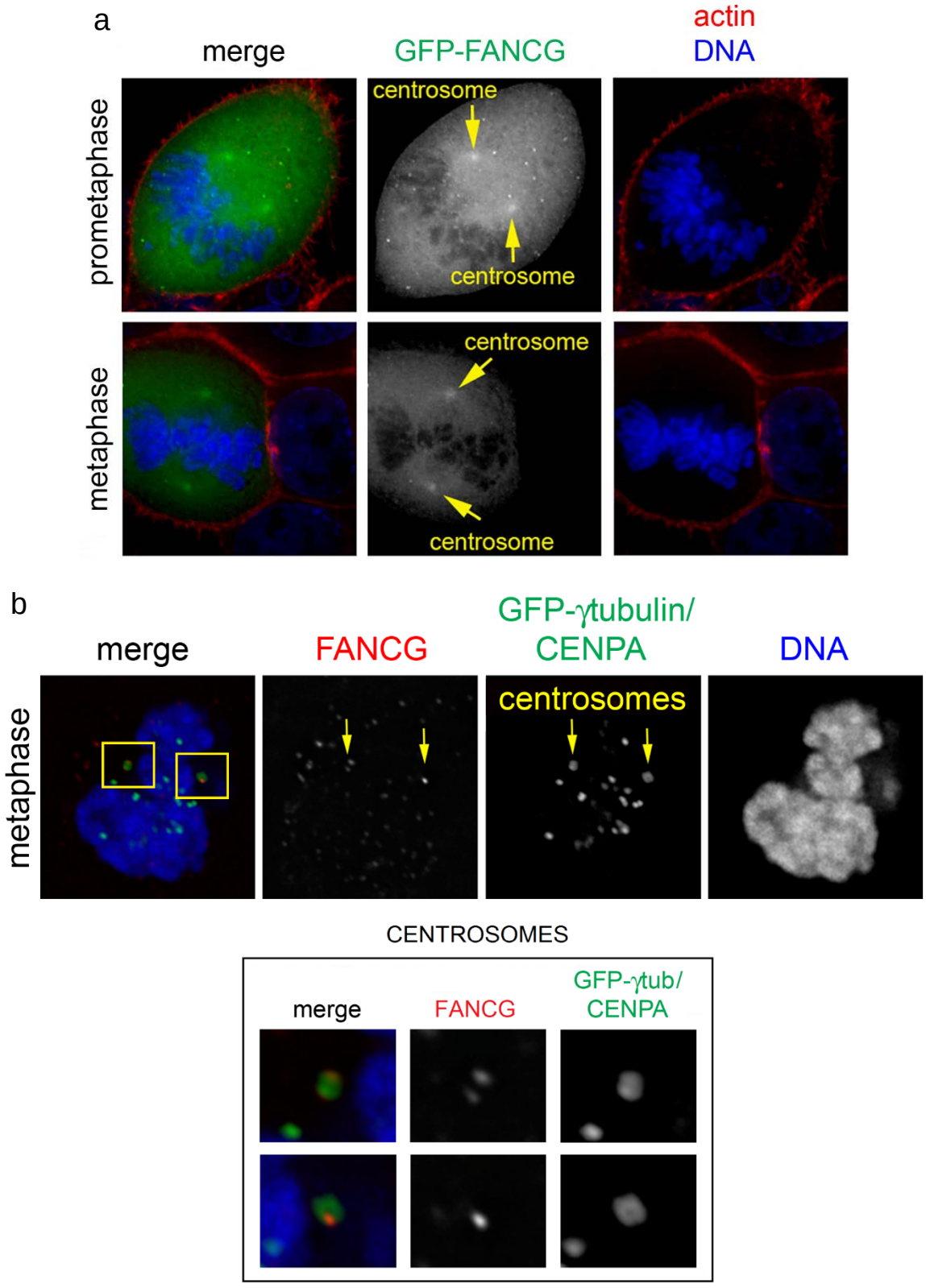


Figure 5-4. Deconvolution microscopy reveals that GFP-fused and endogenous FANCG localizes to the centrosome during mitosis. a) Ectopically expressed GFP-fused FANCG (green) localizes to the centrosomes in prometaphase and metaphase cells. Yellow arrows indicate centrosomes, which are identified based on their morphology and location. Actin staining with AlexaFluor594-conjugated phalloidin (red) outlines cell borders. Original magnification is ×1,000 (Applied Precision personalDV). **b)** Endogenous FANCG (red) co-localizes with GFP-γtubulin (green) at the centrosomes in a metaphase cell. Yellow arrows indicate centrosomes. The enlarged centrosomes below the main panel are the ones enclosed by yellow boxes in the main panel. Original magnification is ×1,000 (Applied Precision personalDV).

GFP-fused FANCL localizes to the centrosome

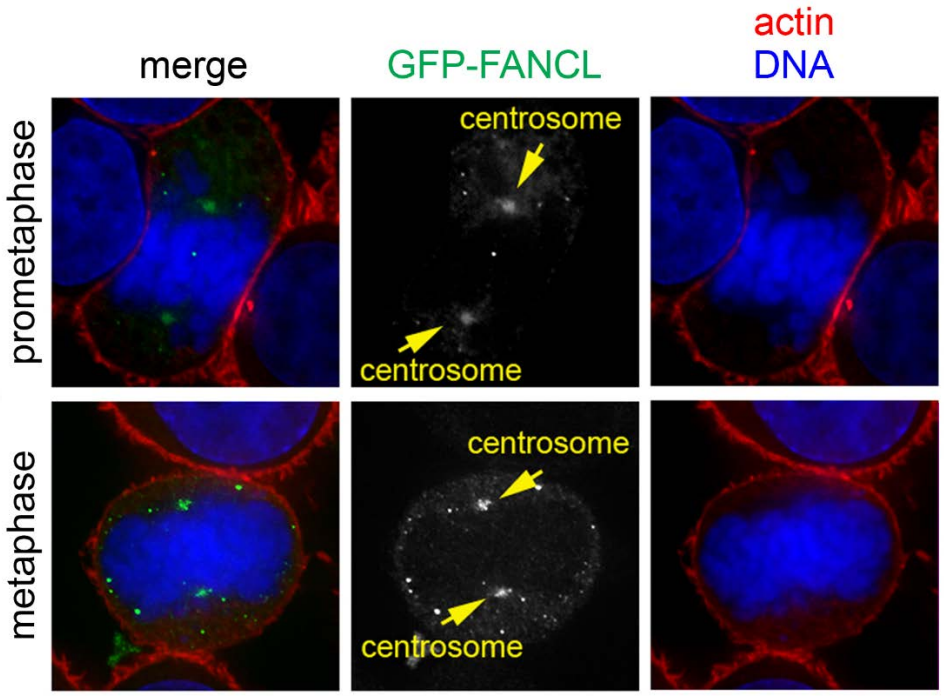


Figure 5-5. Deconvolution microscopy reveals that GFP-fused FANCL localizes to the centrosome during mitosis. Ectopically expressed GFP-fused FANCL (green) localizes to the centrosomes in prometaphase and metaphase cells. Yellow arrows indicate centrosomes. Actin staining with AlexaFluor594-conjugated phalloidin (red) outlines cell borders. Original magnification is ×1,000 (Applied Precision personalDV).

Endogenous FANCD2 localizes to the centrosome

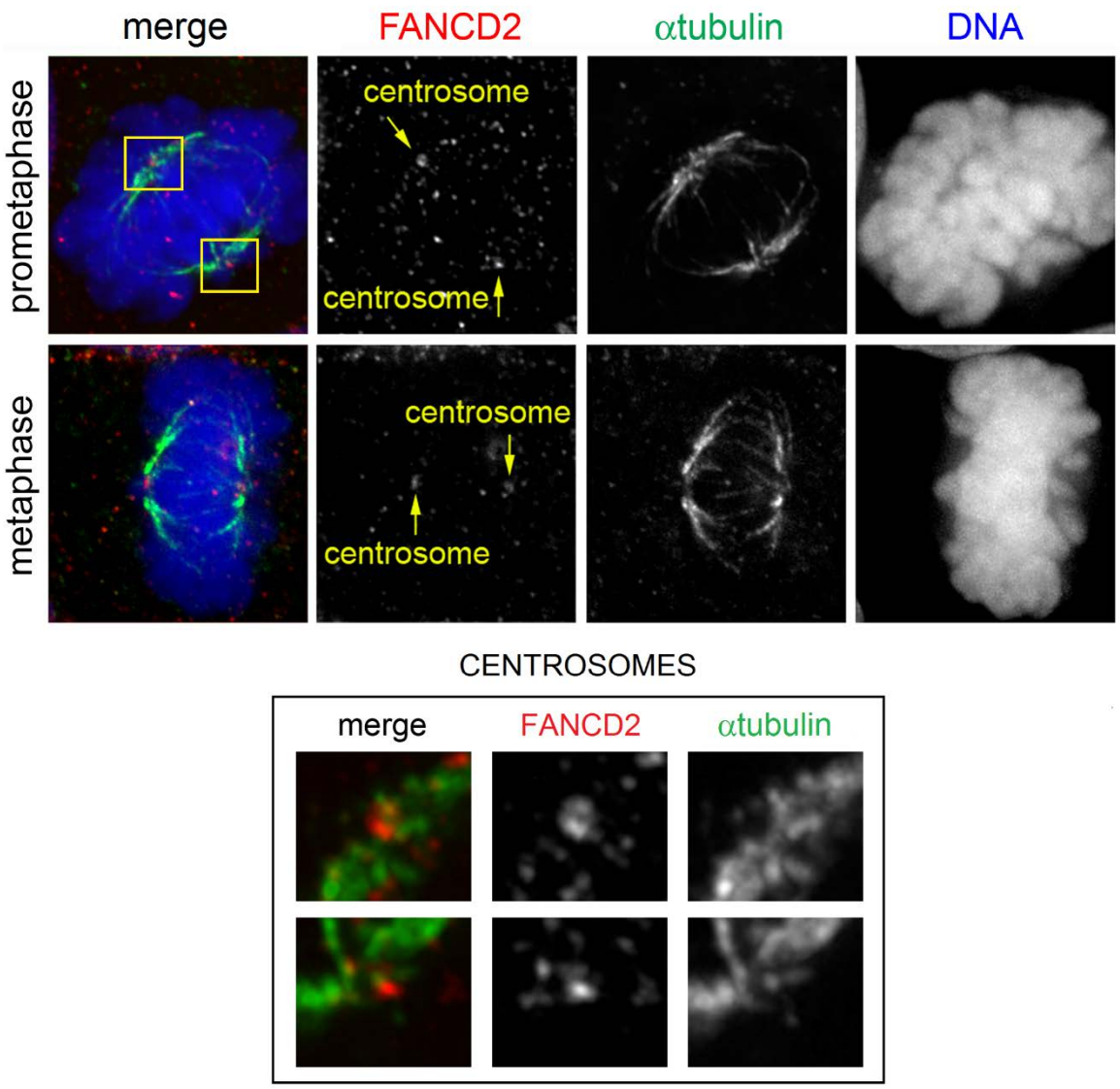
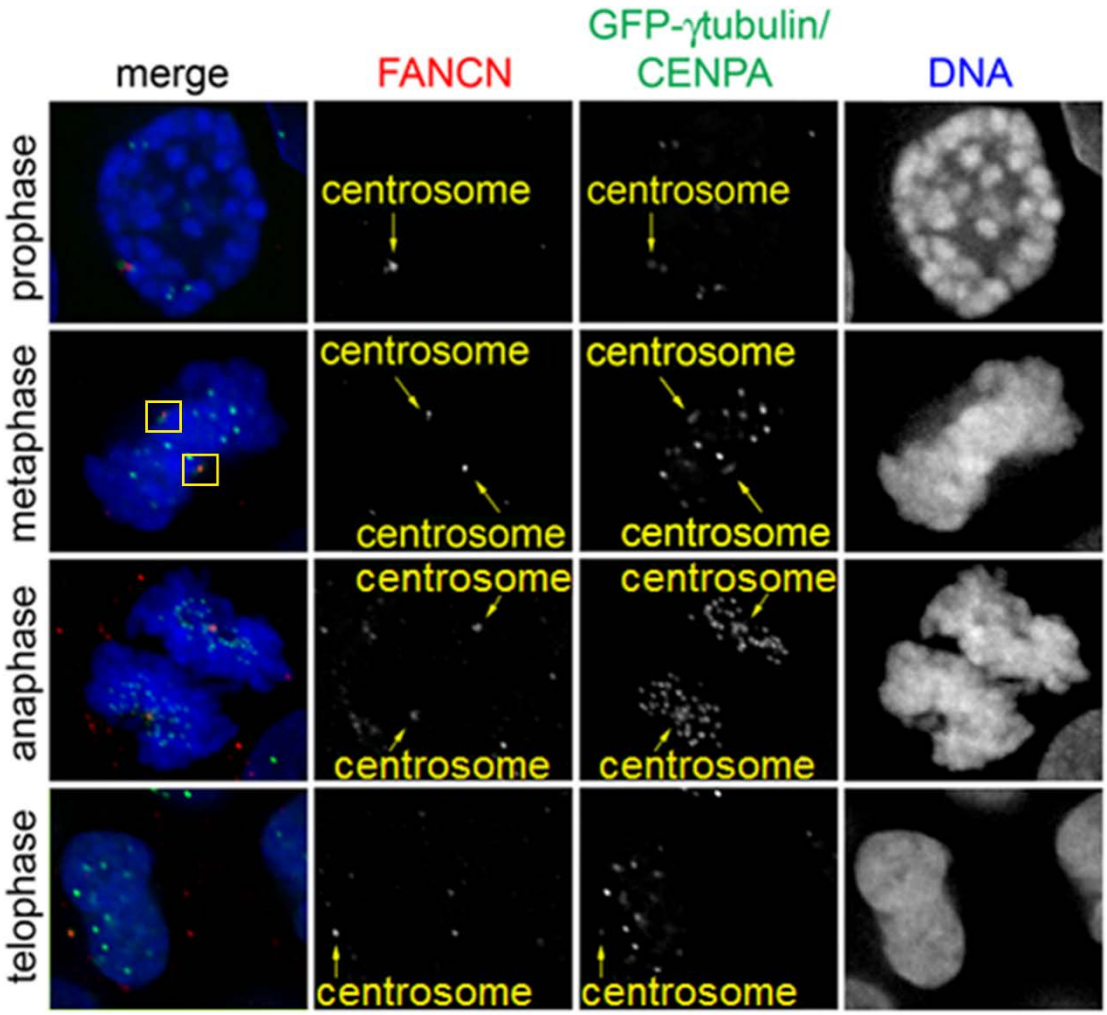


Figure 5-6. Deconvolution microscopy reveals that endogenous FANCD2 localizes to the centrosome during mitosis. Endogenous FANCD2 (red) localizes to the centrosomes in prometaphase and metaphase cells. Co-immunofluorescence staining with αtubulin (green) labels the mitotic spindle. Yellow arrows indicate centrosomes. The enlarged centrosomes below the main panel are the ones enclosed by yellow boxes in the main panel. Original magnification is ×1,000 (Applied Precision personalDV).

Endogenous FANCN/PALB2 localizes to the centrosome



CENTROSOMES

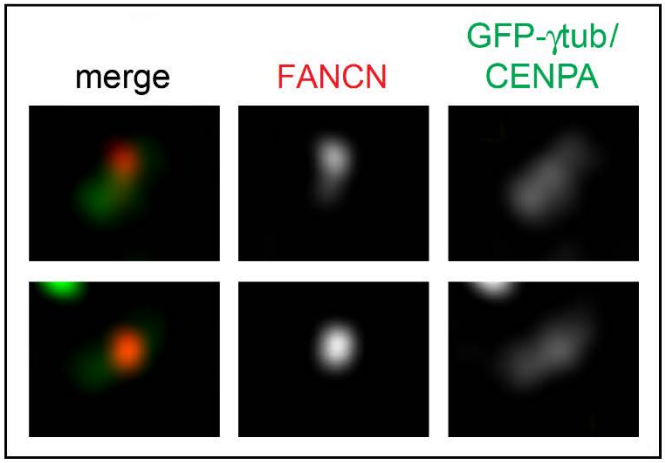


Figure 5-7. Deconvolution microscopy reveals that endogenous FANCN localizes to the centrosome during mitosis. Endogenous FANCN (red) co-localizes with GFP-γtubulin (green) at the centrosomes in prophase, metaphase, anaphase, and telophase cells. Yellow arrows indicate centrosomes. The enlarged centrosomes below the main panel are the ones enclosed by yellow boxes in the main panel. Original magnification is ×1,000 (Applied Precision personalDV).

FANCC localizes to the mitotic spindle, midzone spindle, and to either side of the midbody

We wanted to determine whether FANCC localizes to centrosomes, kinetochores, and/or the mitotic spindle. When immunofluorescence staining of endogenous FANCC was performed in a HeLa cell line stably expressing GFP-fused γtubulin and GFP-fused CENPA, we found that FANCC does not directly co-localize with γtubulin at centrosomes or with CENPA at kinetochores, but rather localizes to the mitotic spindle emanating from the centrosomes during prophase, prometaphase, and metaphase; to the midzone spindle during anaphase; and to either side of the midbody during cytokinesis. To confirm this pattern of localization, co-immunofluorescence staining of FANCC with αtubulin was performed in a HeLa cell line (αtubulin is a microtubule subunit which localizes to the mitotic spindle, midzone spindle, and midbody.) DNA staining was achieved using Hoechst 33342. Localization was analyzed in immunostained cells by deconvolution microscopy. Mitotic cells of each phase were identified based on their nuclear morphology and centrosomal positioning. Mitotic localization of FANCC was further investigated in HeLa cells transfected with ectopically expressed GFP-fused FANCC and studies utilizing biochemical fractionation followed by immunoblotting. Representative microscopy images are shown.

Endogenous FANCC localizes to the mitotic spindle and midzone spindle

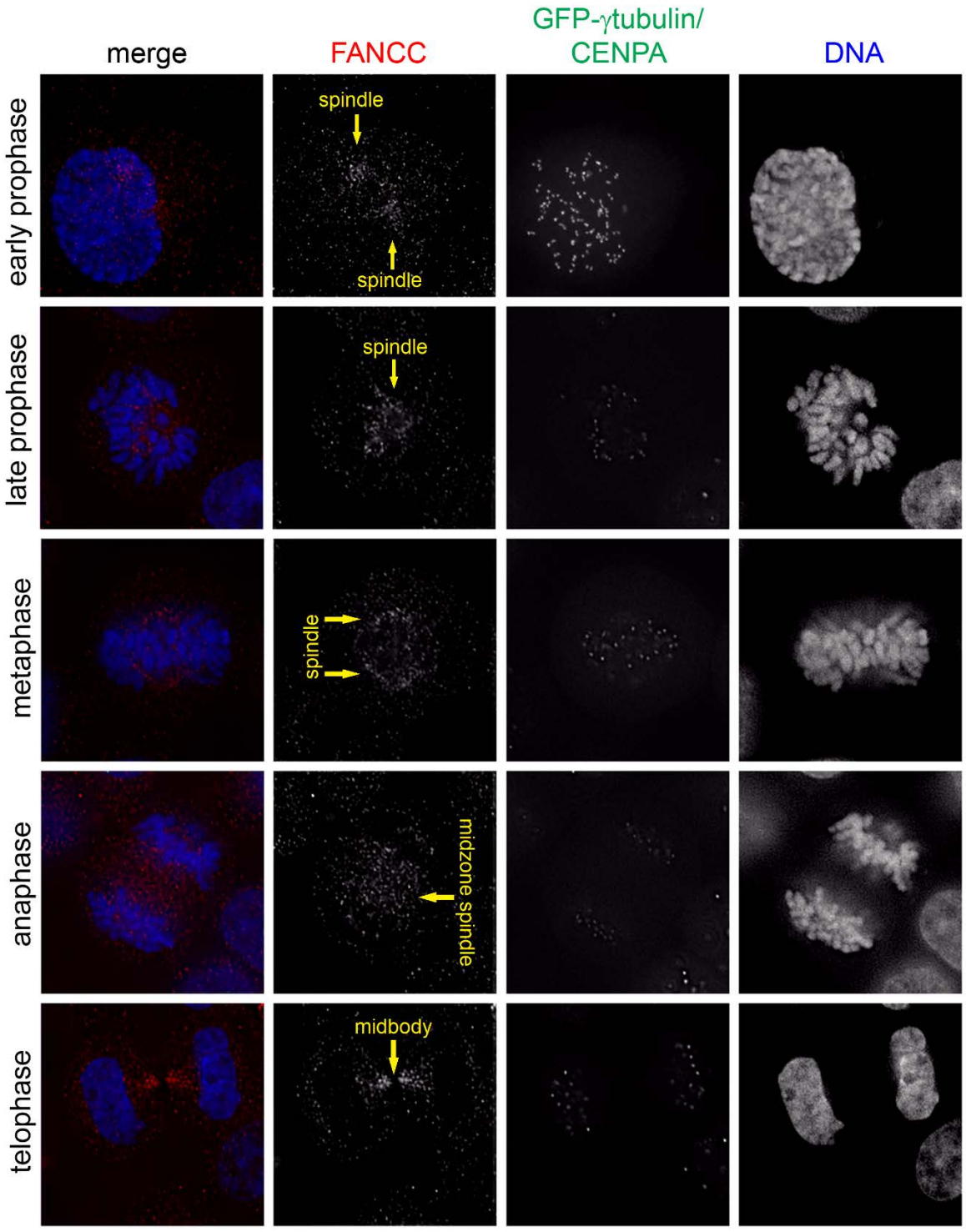


Figure 5-8. Deconvolution microscopy reveals that endogenous FANCC localizes to the spindle in a cell cycle-dependent fashion. Endogenous FANCC (red) localizes to the mitotic spindle in prophase and metaphase, to the midzone spindle during anaphase, and to either side of the midbody during telophase. Original magnification is ×1,000 (Applied Precision personalDV).

Endogenous FANCC co-localizes with α-tubulin during mitosis

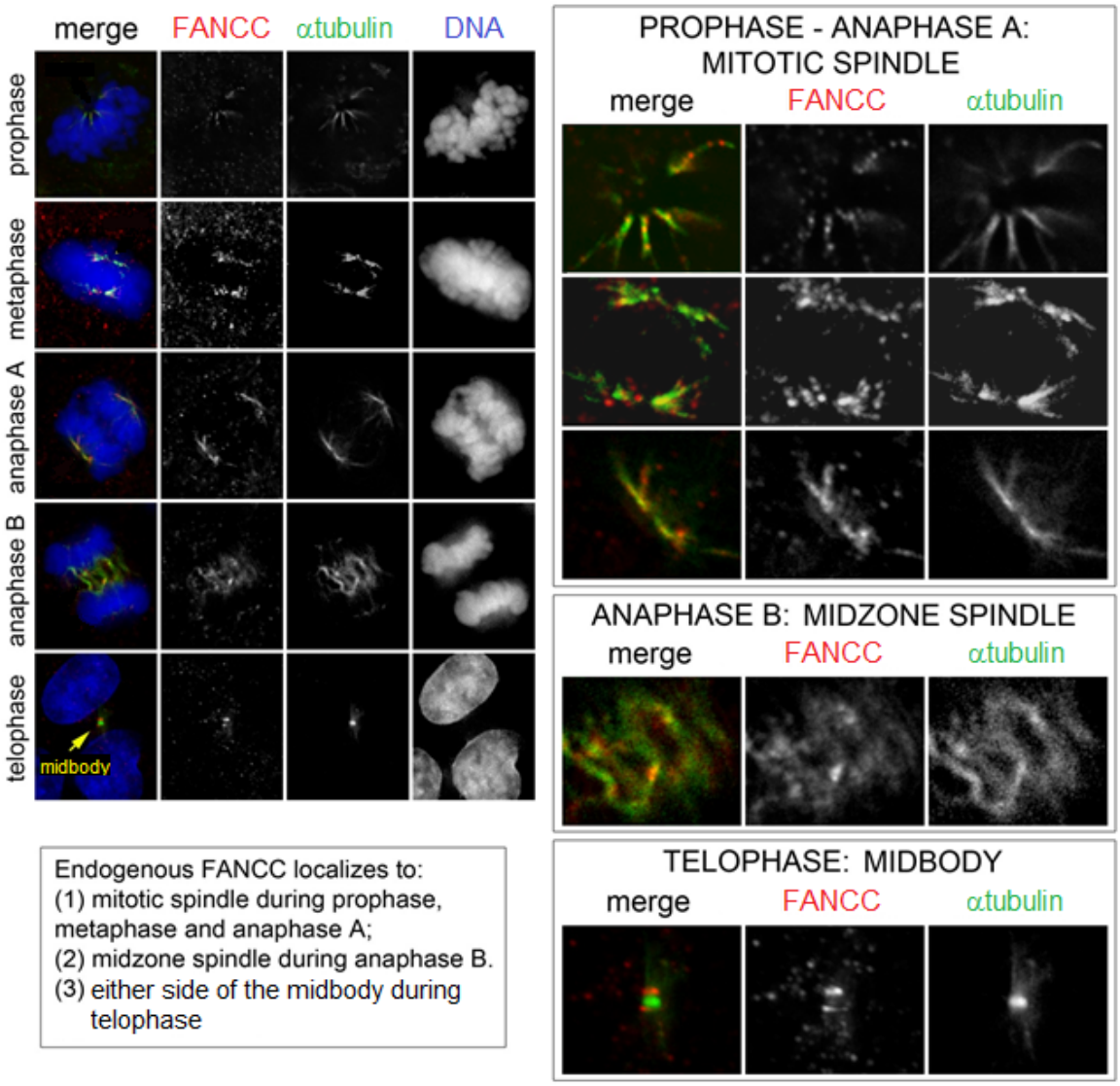


Figure 5-9. Deconvolution microscopy reveals that endogenous FANCC co-localizes with αtubulin on the spindle during mitosis. Endogenous FANCC (red) co-localizes with αtubulin (green) at the mitotic spindle in prophase and metaphase and at the midzone spindle during anaphase. During telophase, αtubulin localizes to the midbody and FANCC localizes to either side of the midbody. Images in the panels on the right are magnified images from the panel on the left. Original magnification is ×1,000 (Applied Precision personalDV).

GFP-fused FANCC localizes to the nucleus during interphase and to the mitotic spindle during mitosis

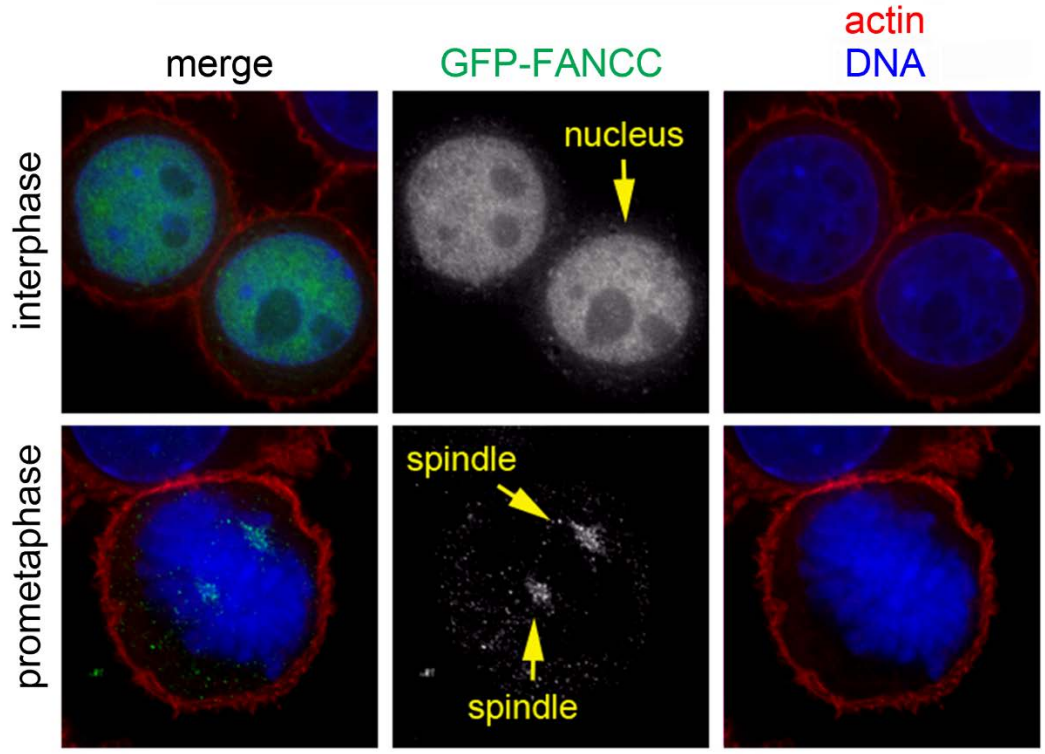


Figure 5-10. Deconvolution microscopy reveals that GFP-fused FANCC localizes to the spindle during mitosis. Ectopically expressed GFP-fused FANCC (green) localizes to the nucleus during interphase and to the spindle during prometaphase. Original magnification is ×1,000 (Applied Precision personalDV).

FANCC localizes to the cytoskeletal subcellular fraction during mitosis

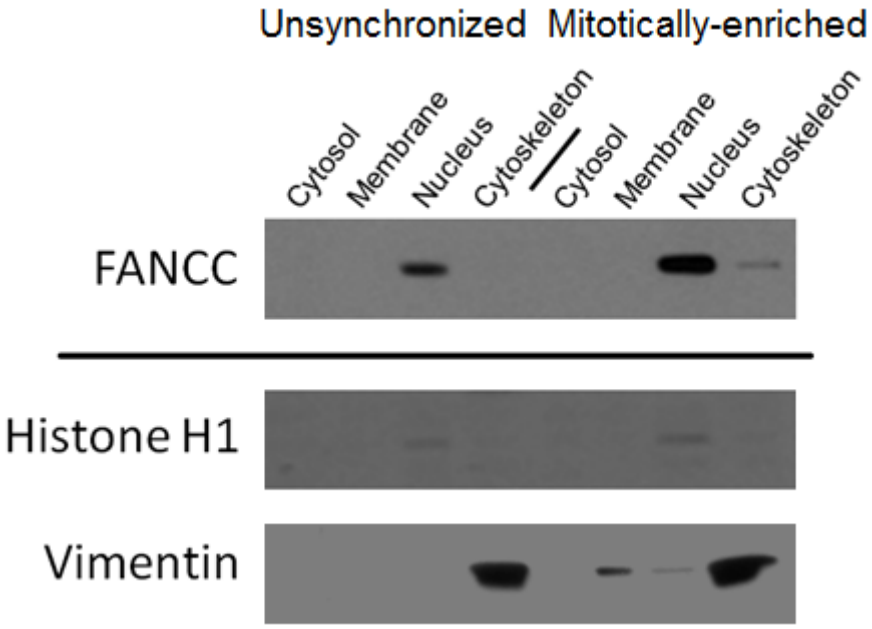


Figure 5-11. Immunoblotting of biochemically fractionated HeLa cells reveals redistribution of FANCC from the nucleus to the cytoskeleton during mitosis. Consistent with microscopy data showing the localization of FANCC to the nucleus in interphase cells, endogenous FANCC is detected in the nuclear fraction of biochemically fractionated unsynchronized HeLa cells. Consistent with microscopy data showing the localization of FANCC to the mitotic spindle, endogenous FANCC is additionally detected in the cytoskeletal fraction of biochemically fractionated HeLa cells following mitotic enrichment. Enrichment of the mitotic cells was achieved by releasing cells from G2 synchronization achieved via treatment with the CDK1 inhibitor RO3306. Histone H1 and vimentin are fractionation controls for the nuclear and cytoskeletal fractions, respectively.

FANCE localizes to kinetochores

To determine whether FANCE localizes to centrosomes, kinetochores, and/or the mitotic spindle, immunofluorescence staining of endogenous FANCE was performed in a HeLa cell line stably expressing GFP-fused CENPA and GFP-fused γtubulin (CENPA is a structural component of the inner kinetochore, and γtubulin is a centrosomal protein). Kinetochores are located on chromosomes at the centromere region, while centrosomes are larger, oval-shaped structures which are located at the mitotic spindle poles. DNA staining was accomplished using Hoechst 33342. Localization was analyzed in immunostained cells by deconvolution microscopy. Mitotic cells of each phase were identified based on their nuclear morphology and centrosomal positioning. FANCE was detected at kinetochores during the early phases of mitosis (prophase, prometaphase, and metaphase) and was absent from kinetochores after the metaphase-to-anaphase transition. This pattern of kinetochore localization is identical to that observed for many key SAC proteins, including the MCC proteins MAD2 and BUBR1. Representative microscopy images are shown.

Endogenous BUBR1 and endogenous FANCE localize to kinetochores

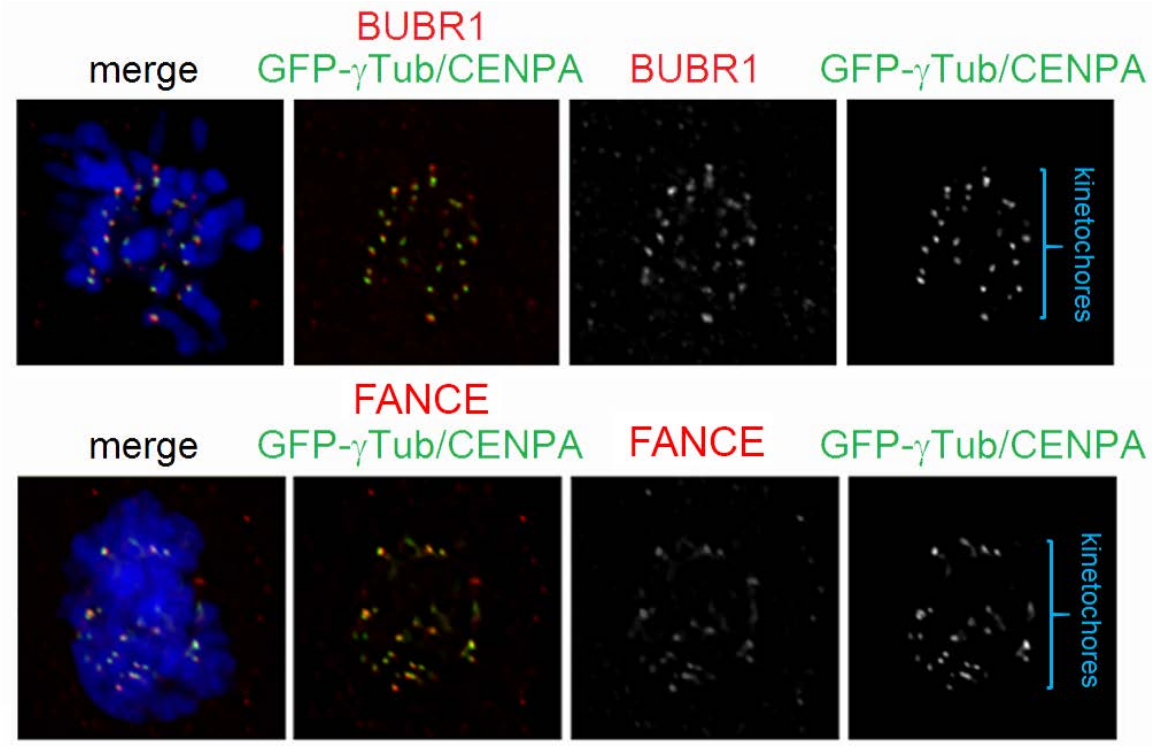


Figure 5-12. Deconvolution microscopy reveals that endogenous FANCE, like endogenous BUBR1, localizes to kinetochores during mitosis. Endogenous BUBR1 (red; top panel) and endogenous FANCE (red; bottom panel) co-localize with GFP-CENPA (green) at the kinetochores in prophase cells. Light blue brackets indicate kinetochores. Original magnification is ×1,000 (Applied Precision personalDV).

Endogenous FANCE localizes to kinetochores during early mitosis

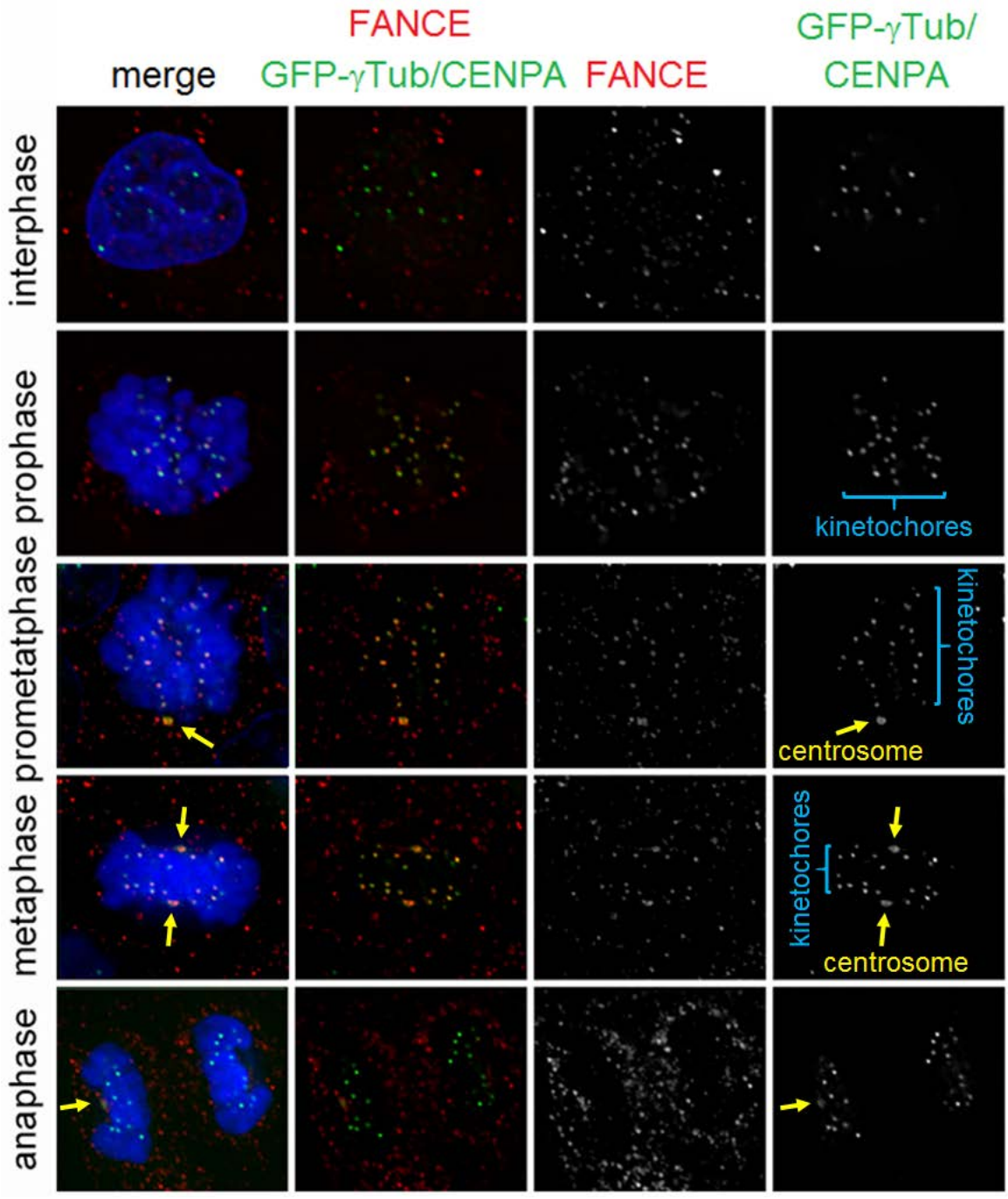


Figure 5-13. Deconvolution microscopy reveals that endogenous FANCE localizes to kinetochores during early mitosis and to centrosomes throughout mitosis. Endogenous FANCE (red) co-localizes with GFP-CENPA (green) at kinetochores during prophase, prometaphase, and metaphase, but not during interphase or anaphase. Endogenous FANCE (red) co-localizes with GFP-γtubulin (green) at the centrosomes throughout mitosis. Light blue brackets indicate kinetochores, and yellow arrows indicate centrosomes. Original magnification is ×1,000 (Applied Precision personalDV).

Summary of the cell-cycle dependent localization of FA proteins to the mitotic apparatus

In summary, the majority of FA proteins localize to the mitotic apparatus in a cell-cycle dependent manner during mitosis. FANCA, FANCB, FANCE, FANCG, FANCL, FANCD2, and FANCN/PALB2 were detected at the centrosomes during mitosis for the first time. Additionally, FANCA and FANCC were found to localize to the mitotic spindle. Finally, FANCE was detected at kinetochores during prophase, prometaphase, and metaphase, but was notably absent from kinetochores during anaphase, telophase, and interphase. These novel sites of localization for the FA proteins are consistent with the newly discovered role of the FA signaling network in the regulation of the mitotic SAC and centrosome maintenance.

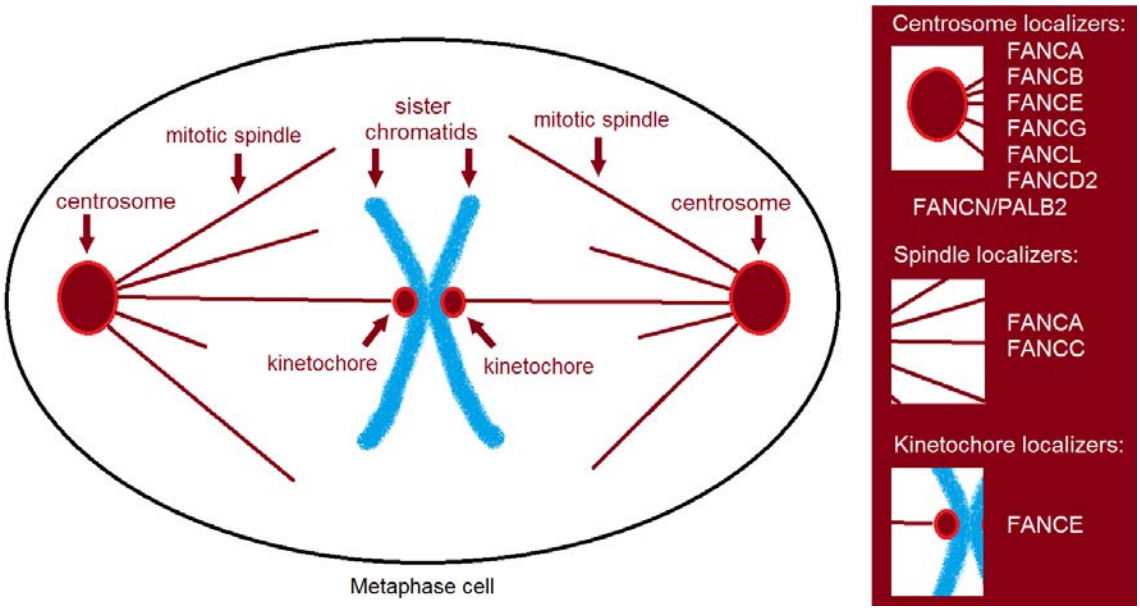


Figure 5-14. Schematic illustrating novel sites of FA protein localization during mitosis identified in this study. We discovered that seven FA proteins localize to mitotic centrosomes, two FA proteins localize to the mitotic spindle, and one FA protein localizes to kinetochores. A diagram of a metaphase cell is shown on the left, with arrows labeling the centrosomes, mitotic spindle, sister chromatids, and kinetochores. A list of the FA proteins which we identified on each part of the mitotic apparatus is shown on the right.

CHAPTER SIX

DISCUSSION

Germline mutations in any one of the known FA genes result in the heterogenous, recessive genetic disease Fanconi anemia. Patients with FA develop congenital malformations, bone marrow failure, and malignancies, especially myeloid malignancies and squamous cell carcinomas. Additionally, mutations in the FA network are etiologically implicated in a significant proportion of inherited pancreatic, breast, and ovarian cancers. The high risk of malignant transformation in FA-deficient cells is due to genomic instability characterized by chromosome breakage and gross aneuploidy. Although it is well established that the FA signaling network plays a central role in the interphase pathway responsible for the repair of DNA interstrand cross-links, the origins of gross aneuploidy in FA-deficient cells are incompletely understood.

The mitotic spindle assembly checkpoint (SAC) is a key tumor suppressor signaling network which protects cells from the development of aneuploidy by ensuring accurate chromosome segregation during mitosis. It is established that FA pathway-deficient cells have a high frequency of aneuploidy and micronucleation, which frequently arise as a result of chromosome mis-segregation. However, the potential role of the FA pathway in the regulation of the mitotic SAC has not previously been analyzed. Recently, our lab generated the first genetically engineered murine model of FA to spontaneously develop bone marrow failure, myelodysplasia, and leukemia, like FA patients do. In this

murine model, as in FA patients, the presence of aneuploidy correlated with the development of MDS and AML. Quantification of the transcriptome in bone marrow from this FA murine model revealed dysregulation of several SAC proteins, including the key MCC proteins Mad2 and BubR1 (A. Pulliam-Leath, S. Ciccone, G. Nalepa, G. Bagby, D.W. Clapp, unpublished data). Therefore, we hypothesized that the FA signaling network plays an essential role in the regulation of the mitotic SAC. My thesis summarizes the results of comprehensive studies addressing this hypothesis.

Establishing an essential role for the FA signaling network in the activity of the mitotic SAC

Initially, we performed a functional RNAi screen to determine whether one or more of the human FA proteins is essential for the activity of the mitotic SAC. Remarkably, SAC failure was observed following taxol challenge for HeLa cells transfected with siRNAs against MAD2 and all FA gene products with the exception of FANCM. The siRNAs used in the screen were successfully validated via immunoblotting for thirteen of the fourteen FA proteins which produced a positive result. We were unable to validate the siRNAs against FANCF and FANCM as commercial antibodies which were tested failed to detect their respective FA protein target. While multiple siRNAs against FANCM produced a negative result in our mitotic SAC screen, these siRNAs remain unvalidated. Furthermore, FANCM is an exceptionally rare FA complementation group for which patient cells are unavailable to researchers. Thus, we were unable to

complete further experiments. However, this study detected an essential role in the activity of the mitotic SAC for all fourteen of the other FA proteins tested.

Off-target effects can produce false-positive results in RNAi screens, and MAD2 has been shown to be particularly susceptible in mitotic RNAi screens. Thus, we tested multiple unique siRNA sequences targeting each FA gene product or we performed quantitative immunoblotting to show that MAD2 is not nonspecifically knocked down by FA siRNAs. These data successfully rule out the possibility that an off-target effect on the level of MAD2 caused the mitotic SAC phenotype for any of the FA siRNAs utilized in our screen.

To confirm the results of the RNAi screen, we assessed the mitotic SAC activity in primary cells from thirteen patients with FA, representing twelve unique FA genotypes. Consistent with the RNAi screen results, primary fibroblasts of all tested FA complementation groups exhibited SAC failure when challenged with taxol. Importantly, genetic correction of primary FANCA patient fibroblasts by stable ectopic expression of FANCA completely rescued the SAC failure following exposure to taxol, confirming that the missing FANCA protein is responsible for the SAC defect in these cells. Next, it was demonstrated that FANCA is required for mitotic SAC arrest in response to nocodazole, a different spindle poison, confirming a general role for the FANCA protein as an essential regulator of the mitotic SAC. Our findings in primary cells from FA patients confirm the results of the siRNA screen and conclusively establish that the FA signaling network plays an essential role in the activity of the human mitotic SAC.

Since FA patients frequently develop aneuploidy-associated hematologic malignancies, we assessed the activity of the mitotic SAC in FANCA-knockdown primary hematopoietic stem and progenitor cells. Weakened activity of the mitotic SAC was observed in taxol-challenged FANCA-knockdown $CD34^+$ cells, indicating that FANCA is essential for the mitotic SAC in human hematopoietic cells. Human $CD34^+$ cells represent early hematopoietic cells, with multipotential capacity for differentiation. When FA patients develop myeloid malignancies, the bone marrow frequently displays clonal hematopoiesis. Thus, myeloid malignancies likely occur in FA patients when a single multipotential hematopoietic cell undergoes malignant transformation and escapes the normal surveillance mechanisms of the hematopoietic cell and its microenvironment. The development of AML in FA patients is generally preceded by the development of complex, random aneuploidy in the bone marrow. Because the mitotic SAC is a major mechanism for the protection of genomic integrity and the prevention of aneuploidy, we propose that weakened activity of the mitotic SAC is a major mechanism contributing to the genesis of aneuploidy and the predisposition to malignant transformation in FA bone marrow.

In summary, an extensive series of functional studies were performed in RNAi-knockdown cells and primary cells from FA patients to examine the activity of the mitotic SAC in the absence of individual FA proteins, definitively establishing an essential role for the FA signaling network in the activity of the mitotic SAC. Of the fifteen FA proteins tested in the RNAi screen, fourteen were found to be essential for the activity of the mitotic SAC. Only FANCM was not

found to be essential for the SAC. Since immunoblotting did not confirm knockdown of FANCM by the three siRNA sequences utilized in our study and since FANCM patient fibroblasts were not available, our study is inconclusive regarding the potential role of FANCM in the activity of the mitotic SAC. However, the literature offers some insight into the potential role of FANCM during mitosis. It is well-established that FANCM plays a key role in FA core complex formation during interphase. FANCM localizes to damaged DNA, promotes the recruitment of the other members of the FA core complex to the nucleus, and acts as a scaffold for the assembly of the FA core complex. However, it was previously shown that FANCM uniquely undergoes proteosomal degradation during the early phases of mitosis (Kee, Kim et al. 2009). Thus, it is possible that FANCM is dispensable in the activity of the mitotic SAC or that degradation of FANCM is essential for mitotic progression. We propose that appropriate regulation of the entire FA signaling network is essential for progression through mitosis and that regulation of the mitotic SAC by the FA signaling network plays a central role in the prevention of chromosome mis-segregation and aneuploidy in dividing cells.

Elucidating the role of FANCA in the activity of the mitotic SAC

We wanted to further understand the role of the FA pathway in the regulation of the mitotic SAC. Thus, a series of studies were designed to systemically examine the role of the FANCA protein in the regulation of the mitotic SAC. Time-lapse microscopy experiments, hypersensitivity assays, and a

mass spectrometry-based screen further illuminated our understanding of the role of the FANCA protein in the regulation of the mitotic SAC.

Time-lapse microscopy of uncorrected and gene-corrected primary FANCA patient fibroblasts was performed in the presence of taxol in order to quantify the duration of SAC arrest and directly visualize the outcome of SAC arrest in FA pathway-deficient cells. FANCA-deficient fibroblasts maintained SAC arrest for a shorter duration and exhibited SAC failure more frequently than complementary gene-corrected fibroblasts. Additionally, time-lapse microscopy of uncorrected and gene-corrected primary FANCA patient-derived fibroblasts was performed in the absence of spindle poisons in order to quantify the duration of mitosis. FANCA-deficient fibroblasts exhibited accelerated progression through prophase and metaphase. Due to weakened SAC, FANCA-deficient cells may initiate anaphase prematurely, without allowing adequate time for the final kinetochore-spindle attachments to form. Thus, accelerated progression through the early mitotic phases is consistent with weakened SAC activity. Collectively, the results of video microscopy experiments in primary FANCA patient fibroblasts confirm that the activity of the mitotic SAC is weakened in FANCA-deficient cells challenged with taxol and that FANCA-deficient cells also exhibit abnormal progression through unperturbed mitosis.

Next, we wanted to know whether FANCA-deficient cells are hypersensitive to drugs targeting the assembly of the mitotic spindle. Thus, uncorrected and gene-corrected primary FANCA patient fibroblasts were challenged with various concentrations of low-dose taxol in a clonogenic assay in

order to determine whether spindle poisons result in decreased survival and proliferation of FA pathway-deficient cells. In this assay, primary FANCA-deficient fibroblasts were hypersensitive to taxol compared with isogenic gene-corrected control fibroblasts. In a previously published assay, it was demonstrated that FANCA-deficient cells are hypersensitive to the spindle poison nocodazole (Kim, Hwang et al. 2013). We additionally performed a survival assay utilizing trypan blue staining and flow cytometry-based cell cycle analysis to characterize the response to low-dose taxol challenge in uncorrected and gene-corrected primary FANCA patient-derived fibroblasts (R. Enzor, G. Hendrickson, G. Nalepa, D. W. Clapp, unpublished data). The doses of taxol utilized in the clonogenic assay produced little to no SAC arrest via flow cytometry-based cell cycle analysis. Thus, the apoptotic response to prolonged SAC arrest is not expected to play a major role in the decreased survival and proliferation of FANCA-deficient cells. Furthermore, the results of hemacytometer-based counts of trypan blue-stained cells indicated that low doses of taxol do not result in increased cell death for FANCA-deficient fibroblasts, compared with isogenic gene-corrected control fibroblasts. Collectively, our results indicated that low doses of taxol result in decreased proliferation, increased cell size, and >4N DNA content in FANCA-deficient fibroblasts. Thus, we propose that the decreased survival and proliferative capacity observed in FANCA-deficient fibroblasts results from the formation of cell-cycle arrested, multinucleated cells.

One of several chemotherapeutic agents targeting the mitotic spindle, taxol is commonly used to treat breast cancer. The role of the FA pathway in the

regulation of the mitotic SAC and the hypersensitivity of FANCA-deficient cells to taxol may partially explain why patients with FA are sensitive to numerous types of chemotherapeutic agents and frequently develop secondary malignancies. We propose that spindle drugs may be useful in non-FA patients who sporadically develop FA-deficient cancers, but that using spindle drugs in FA patients may promote the development of secondary malignancies.

To identify mitotic signaling pathways affected by the loss of FANCA, we designed a proteomics screen to detect altered expression and post-translational modification of mitotic regulators in primary FANCA patient fibroblasts. Following taxol challenge of uncorrected and gene-corrected FANCA patient fibroblasts, total protein levels and phospho-peptide levels were quantified via mass spectrometry. Candidates were defined by a two-fold or greater change in total protein level or phospho-peptide level between the two cell lines. The identified candidates included multiple known regulators of the mitotic SAC. BRCA1 and SKI, two mitotic regulators which are targets of the mitotic kinase Aurora A (AURKA) at the centrosome, have been validated by immunoblotting. The onco-protein SKI is additionally phosphorylated by CDK1, and BRCA1 is known to promote the transcription of MAD2 and BUBR1. Several FA proteins physically interact with CDK1, and it is well-established that the FA pathway interacts with BRCA1 in the repair of DNA damage. Thus, it is likely that BRCA1 and SKI are functionally related to FANCA in the regulation of mitosis. We suggest that BRCA1 and SKI may be up-regulated in FANCA-deficient cells in an attempt to compensate for loss of FANCA.

The FA pathway functionally interacts with two of the key mitotic kinases controlling early mitotic events—CDK1 and PLK1 (Kruyt, Dijkmans et al. 1997, Zou, Tian et al. 2013). Specifically, FANCC plays a role in the regulation of mitotic entry by functioning as an upstream regulator of CDK1 (Kruyt, Dijkmans et al. 1997). A kinase activity assay performed in FANCC siRNA-transfected HeLa cells confirms that FANCC is an upstream regulator of CDK1 (Y. Yang, R. Enzor, G. Nalepa, D. W. Clapp, unpublished data). Additionally, FANCJ activates PLK1 (Zou, Tian et al. 2013). AURKA is a third key mitotic kinase controlling early mitotic events, and the CDK1, PLK1, and AURKA pathways functionally interact and overlap in the control of mitotic entry, spindle assembly, and mitotic progression (Neef, Gruneberg et al. 2007, Lindqvist, Rodriguez-Bravo et al. 2009, Lens, Voest et al. 2010, Van Horn, Chu et al. 2010, Ikeda, Chiba et al. 2012). In our mass spectrometry-based quantification of the proteome and phospho-proteome in primary FANCA patient fibroblasts, multiple candidates were identified which are known targets of the kinases AURKA, CDK1, and PLK1 (see Figure 3-12). Thus, the FA signaling network may regulate mitotic entry and spindle assembly, as well as the mitotic SAC.

Assessing aneuploidy and centrosome amplification as a result of unperturbed mitosis in FA pathway-deficient cells

We have proposed that the essential role of the FA signaling network in the regulation of the mitotic SAC is a key mechanism by which the FA proteins function in the maintenance of genomic stability. Specifically, we hypothesized

that weakened activity of the mitotic SAC in FA-deficient cells promotes abnormal execution of unperturbed mitosis leading to the development of aneuploidy and centrosome amplification. Chromosome mis-segregation due to weakened SAC activity may result in aneuploidy in the form of micronuclei. Furthermore, cytokinesis failure may occur secondary to SAC failure, directly leading to multinucleation and centrosome amplification in FA-deficient cells. A previous study implicating the FA protein FANCD1/BRCA2 in centrosome maintenance and accurate chromosome segregation detected centrosome amplification accompanied by aneuploidy in Brca2-deficient cells (Tutt, Gabriel et al. 1999). Thus, we hypothesized that aneuploidy and centrosome amplification would be present in FA-deficient cells. To assess whether aneuploidy and/or centrosome amplification develop as a result of unperturbed mitosis in FA-deficient cells, fluorescence imaging of RNAi-knockdown cells and primary cells from FA patients was performed.

To determine whether aneuploidy develops as a result of unperturbed mitosis in FA-deficient cells, primary fibroblasts from FA patients of twelve unique FA genotypes were cultured in the absence of spindle poisons. Structural nuclear abnormalities (multinuclei and micronuclei) were observed in primary FA patient fibroblasts of all twelve FA genotypes analyzed. This finding is consistent with previously reported observations of aneuploidy in cells from FA patients. Additionally, centromeres were detected in micronuclei and multinuclei in primary fibroblasts derived from an FA patient of the FANCA subtype. FA-deficient cells exhibit defective DNA damage repair and SAC dysfunction, both of which can

lead to the generation of micronuclei. The presence of centromeres in micronuclei from FANCA patient-derived fibroblasts suggests that these micronuclei developed as a consequence of chromosome mis-segregation, rather than as a result of DNA breakage. Most, but not all, micronuclei observed in the analyzed primary FANCA patient-derived fibroblasts contained centromeres. This finding suggests that chromosome mis-segregation is a major mechanism leading to aneuploidy in FANCA-deficient cells.

Next, we wanted to know if the FA pathway is essential for the maintenance of normal numbers of centrosomes (one or two centrosomes per cell). Since supernumerary centrosomes can promote merotelic kinetochore attachments leading to chromosome mis-segregation, centrosome amplification could additionally contribute to the development of aneuploidy in FA pathway-deficient cells. A description of the relationship between kinetochore attachment and the mitotic SAC and a summary of the role of merotelic kinetochore attachments in the development of aneuploidy are presented below.

To determine whether supernumerary centrosomes develop as a result of unperturbed mitosis in FA-deficient cells, we performed immunostaining of the centrosomal protein pericentrin in FA siRNA-transfected HeLa cells and in primary fibroblasts from FA patients. Centrosome amplification accompanied by multinucleation occured as a result of unperturbed mitosis in HeLa cells transfected with siRNAs against thirteen different FA gene products (all except FANCM and FANCO). Furthermore, an increased percentage of cells with supernumerary centrosomes was observed for all twelve of the FA patient

genotypes tested, and a significant degree of centrosome amplification was observed in primary fibroblasts from nine of the twelve genotypes. Primary fibroblasts from patients with mutations in FANCD2, FANCJ, and FANCN were not significantly different from healthy controls following statistical analysis. However, it is notable that siRNAs against FANCD2, FANCJ, and FANCN resulted in a significant degree of centrosome amplification and that another research group has reported a role for FANCJ in centrosome amplification (Zou, Tian et al. 2013). Thus, we do not rule out a role for FANCD2, FANCJ, and FANCN in centrosome maintenance. In summary, we conclude that FA-deficient cells accumulate supernumerary centrosomes as a result of unperturbed mitosis.

Our study clearly establishes that the absence of a functional FA pathway leads to aneuploidy and centrosome amplification. Our study has identified roles for the FA signaling network in SAC activity and centrosome maintenance and confirms the results of a previous study which observed cytokinesis failure, evidenced by the generation of binucleated cells, in primary FA-deficient murine and human cells (Vinciguerra, Godinho et al. 2010). Weakened SAC and supernumerary centrosomes may result in chromosome mis-segregation leading to the generation of micronuclei, and cytokinesis failure may result in multinucleation. Thus, these represent three novel mechanisms for the development of aneuploidy in FA-deficient cells. This is illustrated in Figure 6-1.

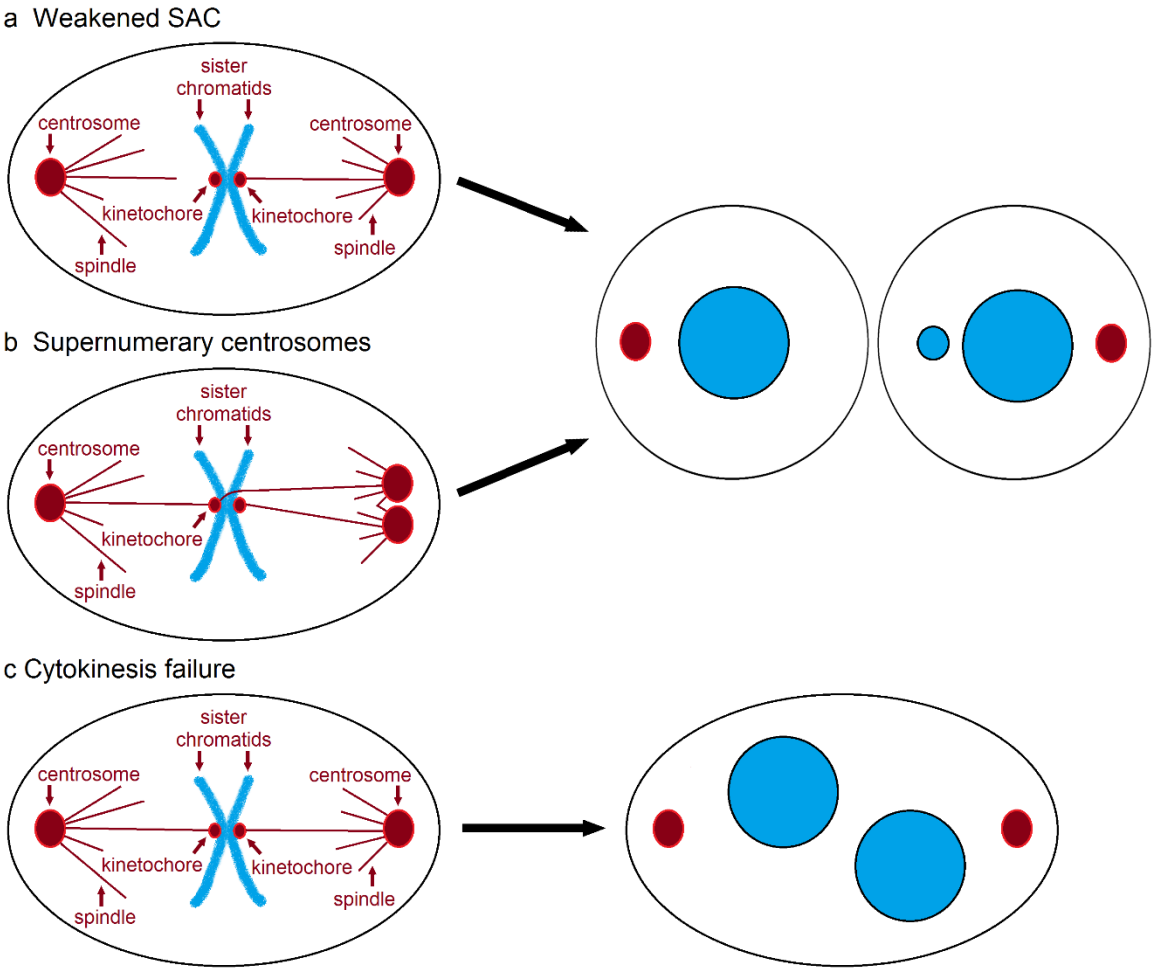


Figure 6-1. Multiple cell cycle defects may lead to aneuploidy in FA-deficient cells. Loss of FA pathway activity results in the development of aneuploidy due to **a)** weakened SAC, **b)** presence of supernumerary centrosomes, and **c)** cytokinesis failure. Centrosomes and kinetochores are shown in red, and DNA is shown in blue.

It is unclear whether cytokinesis failure secondary to abnormal SAC regulation is the main source of centrosome amplification in FA-deficient cells or whether primary defects in centrosome replication and/or cytokinesis also contribute to this phenotype. When centrosomes were quantified in FA-deficient cells, we noted that the presence of centrosome amplification is frequently accompanied by the presence of aneuploidy in FA-deficient cells. This finding suggests that the presence of supernumerary centrosomes may be occurring as a result of cytokinesis failure secondary to SAC dysfunction. This possibility is discussed in greater detail toward the end of the current chapter. Additionally, experiments are proposed in the Future Directions to investigate the potential role of the FA signaling network in centrosome replication and to determine whether centrosome amplification in FA-deficient cells is due to a primary defect in centrosome replication or is occurring secondarily to SAC dysfunction.

Proper kinetochore attachment to the mitotic spindle is the key event which satisfies the SAC. Types of kinetochore-spindle fiber attachment are summarized in Figure 6-2. Normally, monotelic attachment of one kinetochore to spindle fibers from a single spindle pole occurs during early prometaphase (Figure 6-2a), and amphitelic (bipolar) attachment follows as the second sister chromatid's kinetochore attaches to spindle fibers from the opposite spindle pole (Figure 6-2b). The presence of a syntelic kinetochore attachment activates the mitotic SAC since the other kinetochore remains unattached (Figure 6-2c). However, the presence of a merotelic kinetochore attachment escapes detection by the mitotic SAC since both kinetochores are attached (Figure 6-2d).

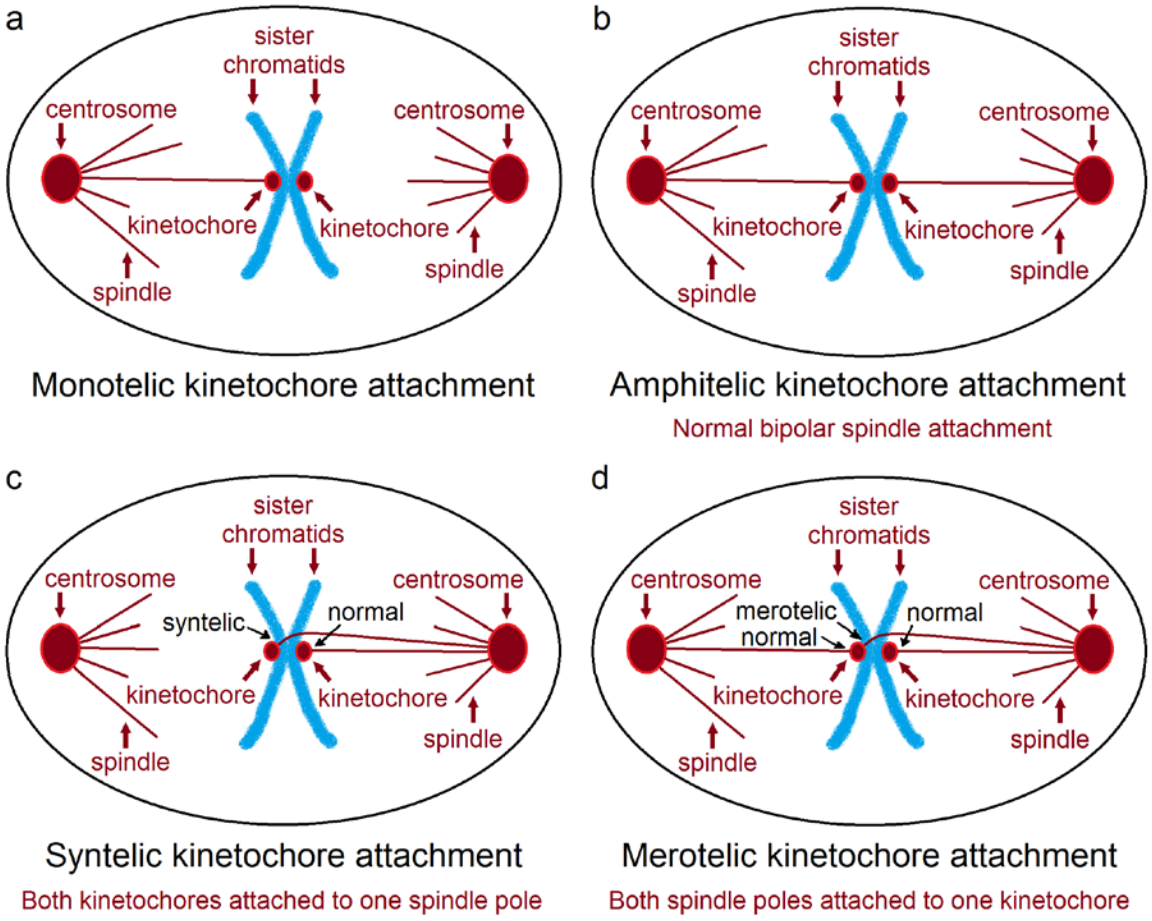


Figure 6-2. Schematic summarizing the types of kinetochore attachment.

As previously mentioned, supernumerary centrosomes can result in the formation of a multipolar mitotic spindle leading to merotelic kinetochore attachments. Due to the stochastic (random) nature of kinetochore-spindle fiber attachments, merotelic kinetochore attachments may form during early prometaphase, but the cell has mechanisms for correcting them (Cimini, Moree et al. 2003, Pinsky, Kung et al. 2006, Cimini 2007, Maure, Kitamura et al. 2007, Silkworth and Cimini 2012). The mitotic kinase Aurora B and other mitotic regulators respond to the presence of merotelic attachments by destabilizing them. Turning kinetochores with merotelic attachments into unattached kinetochores activates the mitotic SAC and allows the dividing cell to form amphitelic attachments prior to the initiation of anaphase (Pinsky, Kung et al. 2006, Cimini 2007, Maure, Kitamura et al. 2007).

Since the SAC may be satisfied in the presence of merotelic kinetochore attachments, states promoting the development of merotelic attachments may result in chromosome mis-segregation leading to aneuploidy. Merotelic kinetochore attachments may develop as a result of supernumerary centrosomes through a model summarized in Figure 6-3 which was originally proposed by another research group (Ganem, Godinho et al. 2009). Since merotelic kinetochore attachments may result in chromosome mis-segregation, supernumerary centrosomes promote the development of aneuploidy.

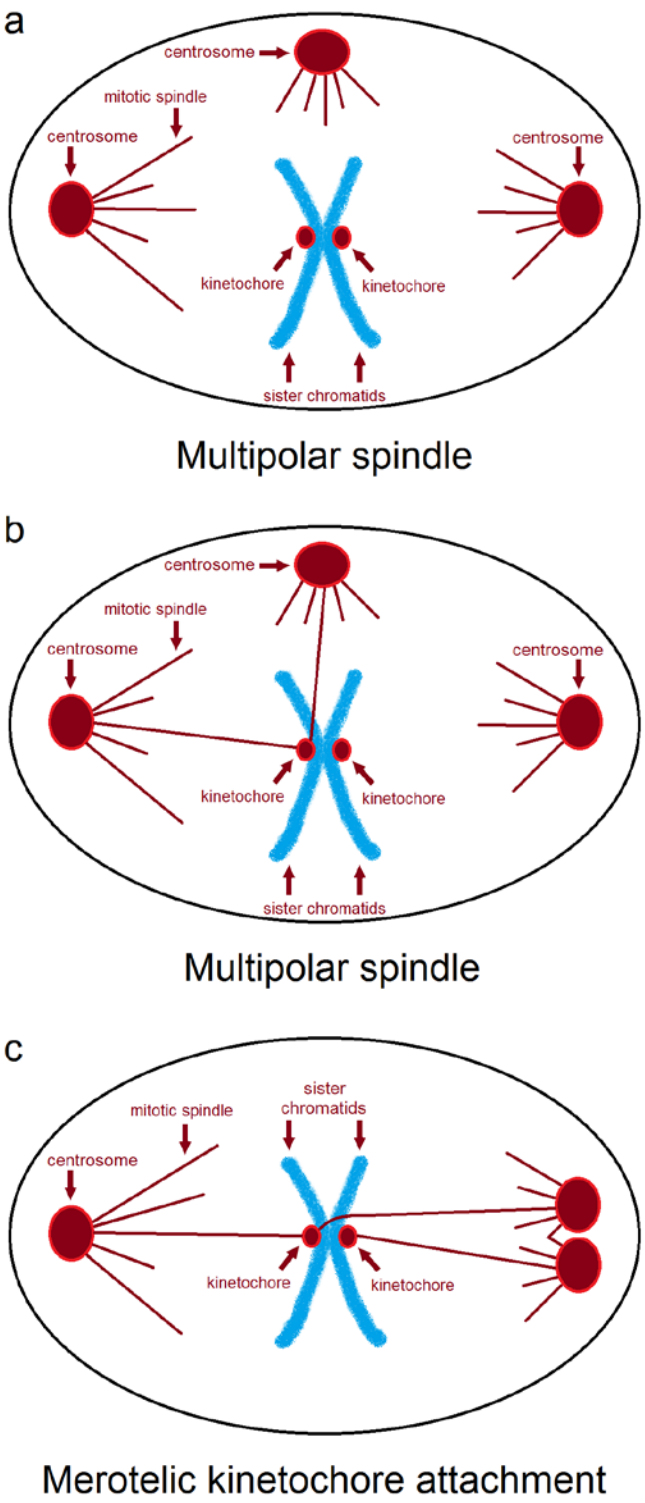


Figure 6-3. Schematic illustrating the development of merotelic kinetochore attachment in a cell with a multipolar spindle. a) Supernumerary centrosomes lead to the formation of a multipolar spindle. **b)** Kinetochore-spindle attachments form in the context of a multipolar spindle. **c)** Supernumerary centrosomes migrate to two spindle poles, resulting in merotelic attachment.

It is unclear whether primary defects in the regulation of cytokinesis exist in FA pathway-deficient cells. When the outcome of prolonged SAC arrest was analyzed by live-cell video microscopy of taxol-challenged primary FANCA patient fibroblasts, cytokinesis failure followed SAC failure in the majority of cells, and the rate of cytokinesis failure was identical in FANCA-deficient fibroblasts and gene-corrected control fibroblasts. Thus, our data suggests that abnormal SAC regulation may be responsible for the cytokinesis failure occurring in FA-deficient cells. However, phenotypic characterization via live-cell video microscopy of untreated primary FANCA patient fibroblasts revealed defects in endocytic regulation during cytokinesis. Additionally, FANCC localizes to the midzone spindle during anaphase and to either side of the midbody during telophase, a pattern resembling that of known regulators of cytokinesis. Additional experiments will be necessary to examine the potential roles of FANCA, FANCC, and other FA proteins in the regulation of cytokinesis. These are presented in the Future Directions (Chapter Seven).

Novel mitotic abnormalities in primary FANCA patient fibroblasts

Time-lapse microscopy of uncorrected and gene-corrected primary FANCA patient fibroblasts was performed in the absence of spindle poisons in order to phenotypically characterize unperturbed mitosis in the absence of FANCA. FANCA-deficient fibroblasts exhibited a variety of phenotypic defects suggesting defective chromosome congression (inability to form metaphase plates), mis-orientation of the mitotic spindle, abnormal nuclear envelope

regulation, and abnormal endocytosis. These results indicate novel mitotic abnormalities in FANCA-deficient cells. Experiments to validate the potential role of FANCA in each of these processes are described in the Future Directions.

The FA signaling network has previously been shown to interact with pathways regulating each of these processes. It was recently discovered that the FA signaling network regulates the WNT/β-catenin pathway, a pathway that plays a key role in specification of stem cell fate by controlling the orientation of the mitotic spindle. The phenotype of spindle orientation and the connection between the FA signaling network and the WNT/β-catenin pathway are discussed in the next subsection. Additionally, FANCA physically interacts with HTT (Huntingtin), a protein known to regulate endocytosis and spindle orientation. Furthermore, HTT is a known effector of Rab5, a GTPase which regulates mitotic nuclear envelope remodeling and congression of chromosomes to the metaphase plate. The potential link between FANCA and HTT is discussed in a later subsection.

Spindle mis-orientation

When unperturbed mitosis was examined in primary FANCA-deficient patient fibroblasts via time-lapse microscopy, spindle mis-orientation was observed. Proper orientation of the mitotic spindle is necessary for events in embryogenesis and organogenesis, and defects in cell polarity and orientation of the dividing cell have been linked to numerous developmental problems (Zigman, Cayouette et al. 2005, Wilcock, Swedlow et al. 2007, Segalen and Bellaiche 2009, Godin, Colombo et al. 2010, Pulvers, Bryk et al. 2010, El-Hashash,

Turcatel et al. 2011, Morin and Bellaiche 2011, Bellis, Duluc et al. 2012, Bubenshchikova, Ichimura et al. 2012, Noatynska, Gotta et al. 2012, Fujimori, Itoh et al. 2013, Williams and Fuchs 2013, Elias, Thion et al. 2014, Hell, Duda et al. 2014). Since FA patients develop microcephaly and malformations of the kidneys and digestive system (Alter 2008), it is notable that mis-orientation of the mitotic spindle has been linked to genes implicated in congenital microcephaly and in kidney and intestinal abnormalities (Shen, Eyaid et al. 2005, Higgins, Midgley et al. 2010, Quyn, Appleton et al. 2010, Bellis, Duluc et al. 2012, Bubenshchikova, Ichimura et al. 2012, Hell, Duda et al. 2014).

FA results in a broad array of congenital malformations, most commonly "skin hyperpigmentation and *café au lait* spots; short stature; abnormal thumbs and radii; abnormal head, eyes, kidneys, and ears" (Alter 2008). The gonads, digestive organs, and heart may also be affected. Approximately 75% of FA patients have notable congenital defects, which can aid clinicians by increasing the diagnostic suspicion of FA (Alter 2008). The congenital malformations which are known to occur in patients with FA are summarized in Table 6-1 below. We propose that mis-orientation of the mitotic spindle may be responsible for the development of congenital abnormalities in FA patients.

Organ systems	Organs	Most common defect	Other defects
Integumentary	Skin	hyperpigmen-tation	*café au lait* spots
			hypopigmentation
Skeletal	Body	short stature	
	Thumbs	absent or hypoplastic thumbs	bifid or triphalangeal thumbs
			rudimentary thumbs
			thumbs attached by a thread
	Arms & legs	absent or hypoplastic radii	dysplastic ulnae
			congenital hip dislocation
			congenital high scapula
	Hands & feet	hypoplastic thenar eminence	absent first metacarpal
			toe syndactyly
			abnormal toes
	Head & face	microcephaly	micrognathia
			triangular face
	Neck & spine	fused cervical vertebrae	spina bifida
			scoliosis
			abnormal ribs
Reproductive	Male	hypogenitalia	undescended testes
			micropenis
	Female	hypogenitalia	bicornuate uterus
			abnormal menses
Sensory	Eyes	small or close-set eyes	strabismus
			epicanthal folds
			cataracts
			astigmatism
	Ears	deafness (conductive deficit)	abnormal shape
			atresia
			abnormal middle ear
Urinary	Kidneys	ectopic or pelvic kidneys	mis-shapen or horseshoe
			hypoplastic or dysplastic
			absent kidneys
			hydronephrosis
	Ureters	hypospadias (male) or megaureter	
Digestive	Esophagus	atresia	tracheoesophageal fistula
	Intestines	atresia	imperforate anus
Circulatory	Heart	various congenital structural defects	
	Vasculature	weak or absent radial pulse	

Table 6-1. Summary of congenital defects in FA patients. FA patients develop a wide variety of congenital malformations in organ systems throughout the human body. Skeletal abnormalities and defects in skin pigmentation are the most common, but FA patients may also have defects in the reproductive, sensory, urinary, digestive, and circulatory organs. This table was adapted from the list of “Examples of Anomalies in Fanconi Anemia” in the Fanconi Anemia Guidelines for Diagnosis and Management, Third Edition, 2008, pp. 34-35 (Alter 2008).

WNT/β-catenin signaling is essential for spindle orientation and cell fate specification and plays roles in embryogenesis, development of multiple organ systems, and maintenance of the hematopoietic stem cell pool (Walston, Tuskey et al. 2004, Nemeth and Bodine 2007, Kim, Kang et al. 2009, Oh 2010, Buchman, Durak et al. 2011, Perry, He et al. 2011, Ruiz-Herguido, Guiu et al. 2012). Abnormalities in WNT signaling have been linked to developmental abnormalities and the induction of cancer (Moon, Kohn et al. 2004, Klaus and Birchmeier 2008, Paul and Dey 2008, MacDonald, Tamai et al. 2009). In the mass spectrometry-based quantification of the proteome and phospho-proteome in uncorrected and gene-corrected primary FANCA patient fibroblasts, we identified numerous candidates related to WNT/β-catenin signaling. Furthermore, it was recently discovered that the FA signaling network regulates the WNT/β-catenin signaling pathway. FANCL ubiquitinates β-catenin and thereby "increases the activity and expression of β-catenin, a key pluripotency factor in hematopoietic stem cells" (Dao, Rotelli et al. 2012). Additionally, FANCC and CtBP1 form a complex with β-catenin, and they negatively regulate expression of DKK1, an inhibitor of WNT signaling. In the absence of FANCC, DKK1 is overproduced, a state known to promote the development of hematopoietic malignancies (Niida, Hiroko et al. 2004, Huard, Tremblay et al. 2013, Huard, Tremblay et al. 2014). Thus, regulation of the WNT/β-catenin signaling pathway by the FA signaling network may provide an explanation for spindle mis-orientation, developmental abnormalities, and bone marrow failure in FA-deficient cells.

Additional experiments will be necessary to characterize the phenotype of spindle mis-orientation in FA-deficient cells and to establish a connection between spindle orientation and developmental abnormalities in FA. Furthermore, additional studies characterizing WNT/β-catenin signaling in FA-deficient cells should be performed, and a murine model should be developed to investigate the potential connection between WNT/β-catenin signaling and the phenotypes of developmental abnormalities, hematopoietic stem cell exhaustion, and cancer predisposition in the context of FA. A more thorough discussion of potential experiments is presented in the Future Directions.

Other abnormal phenotypes

When unperturbed mitosis was examined in uncorrected and gene-corrected primary FANCA patient-derived fibroblasts via time-lapse microscopy, severe abnormalities in vesicle trafficking were observed in FANCA-deficient cells. This finding suggests that FANCA may play a role in the endocytic pathways which are essential for cleavage furrow formation during cytokinesis. Notably, FANCA has previously been found to physically interact with HTT (huntingtin), the protein responsible for Huntington's Disease. HTT has previously been shown to regulate spindle orientation, endocytosis, and neurodevelopment (Pal, Severin et al. 2006, Pal, Severin et al. 2008, Feng 2010, Godin, Colombo et al. 2010, Godin and Humbert 2011b). HTT has additionally been implicated in the division and differentiation of mammary stem cells (Elias, Thion et al. 2014). HTT is an essential regulator of Rab11, a GTPase which

regulates endocytosis and is essential for cleavage furrow formation and abscission during cytokinesis (Tarbutton, Peden et al. 2005, Wilson, Fielding et al. 2005, Power, Srinivasan et al. 2012). Additionally, HTT is an effector of Rab5, a GTPase which regulates lamin disassembly and chromosome congression during early mitosis and also regulates endocytosis (Pal, Severin et al. 2006, Pal, Severin et al. 2008, Capalbo, D'Avino et al. 2011, Serio, Margaria et al. 2011, Lanzetti 2012). HTT was identified as a candidate in the mass spectrometry-based quantification of the proteome and phospho-proteome in uncorrected and gene-corrected primary FANCA patient fibroblasts. Furthermore, live-cell video microscopy of primary FANCA patient fibroblasts revealed potential defects in spindle orientation, chromosome congression, nuclear envelope regulation, and vesicle trafficking during cell division. Since HTT, Rab5, and Rab11 have been implicated in all of these processes, we suggest that FANCA and HTT may functionally interact in the regulation of mitosis and cytokinesis. Further studies will be necessary to validate our previous results and to systematically investigate this hypothesis. These are described in the Future Directions.

Localization of FA proteins to the mitotic apparatus

Our study identified novel roles for the FA signaling network in the regulation of the mitotic SAC and centrosome maintenance. Thus, we hypothesized that FA proteins would localize to the centrosomes, kinetochores, and mitotic spindle. We discovered that seven FA proteins—FANCA, FANCB, FANCE, FANCG, FANCL, FANCD2, and FANCN/PALB2—localize to the

centrosome in a cell-cycle dependent fashion. It has previously been demonstrated that FANCD1/BRCA2 localizes to the centrosome during mitosis. When another group examined the centrosomal localization of FA proteins, their findings confirmed that FANCA, FANCB, and FANCG localize to the centrosome and additionally demonstrated that FANCM, FANCI, and FANCJ localize to the centrosome (Zou, Tian et al. 2013). (See Figure 6-3 below for a schematic summarizing the FA proteins which have been discovered to localize to the mitotic apparatus, including the centrosomes, kinetochores, and mitotic spindle.)

We discovered that FANCA additionally localizes to the mitotic spindle and FANCE additionally localizes to kinetochores. It has previously been demonstrated that FANCD1/BRCA2 localizes to kinetochores during mitosis (Choi, Park et al. 2012), and our experiments confirm this observation (data not shown). Notably, FANCE and FANCD1/BRCA2 localize to kinetochores only from prophase to metaphase and are absent from the centromere region of DNA during interphase and after the metaphase-to-anaphase transition. This pattern of kinetochore localization is consistent with a role in the mitotic SAC. Finally, FANCC was detected on the mitotic spindle and midzone spindle in a unique pattern resembling that of several regulators of anaphase spindle dynamics and cytokinesis. Unlike other FA proteins, FANCC did not co-localize with γtubulin at the centrosome, but rather localized to spindle fibers surrounding the centrosome. Our localization studies revealed that the majority of the FA proteins are found on the mitotic apparatus during cell division, consistent with a role for the FA pathway in the activity of the mitotic SAC.

We hypothesized that FA proteins would localize to the mitotic apparatus in a cell cycle-dependent manner, reflecting the newly established role of the FA signaling network in the regulation of the mitotic SAC. As expected, twelve FA proteins were detected on the mitotic apparatus in studies by our research group and others. Interestingly, eleven FA proteins have been detected at the centrosome, two FA proteins have been detected at the kinetochore, and two FA proteins have been detected at the mitotic spindle. The known sites of FA protein localization during mitosis are summarized in Figure 6-4. Future studies may identify additional FA proteins at each of these sites.

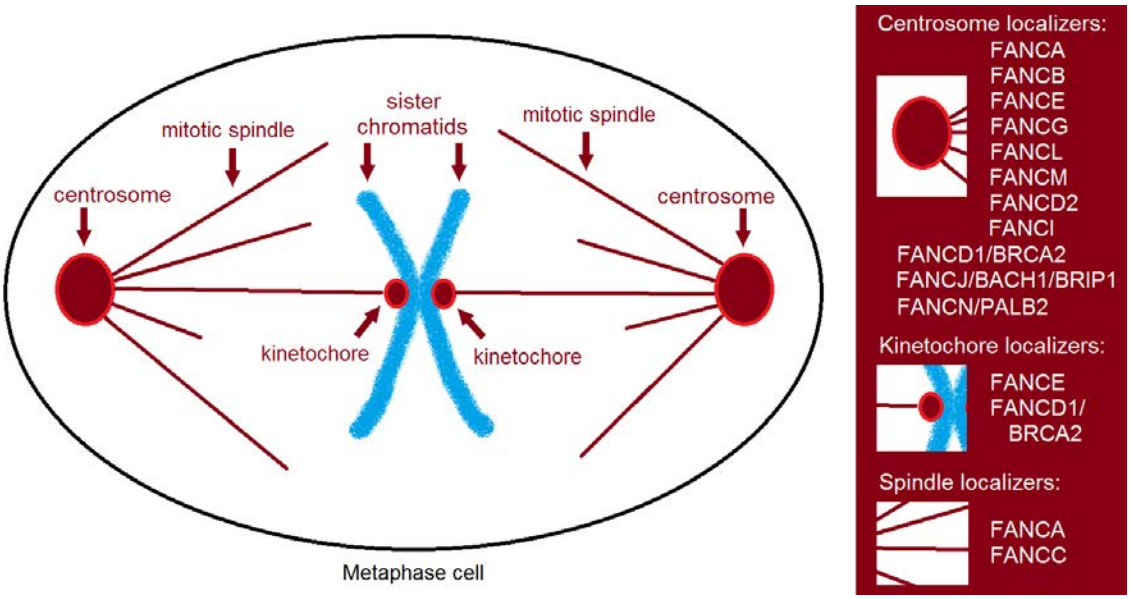


Figure 6-4. FA proteins localize to the mitotic spindle, centrosomes, and kinetochores during cell division. In studies by our research group and others, a total of twelve FA proteins have been detected on the mitotic apparatus. Eleven FA proteins have been detected at centrosomes, two FA proteins at kinetochores, and two on the mitotic spindle.

The groups of FA proteins localizing to each part of the mitotic apparatus do not correspond to known complexes and subcomplexes of FA proteins which exist during interphase. In response to DNA damage during interphase, eight of the FA proteins—FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM—form a structure termed the FA core complex, which functions as a multisubunit ubiquitin ligase. It has been shown that FANCA/FANCG, FANCC/FANCE, and FANCB/FANCL form subcomplexes during core complex assembly (Hodson and Walden 2012). Once assembled in the nucleus, at the site of DNA damage, the core complex monoubiquitinates FANCD2 and FANCI. The FANCD2/FANCI heterodimer signals to a breast-cancer associated set of downstream effectors, including the FA proteins FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, and FANCO/RAD51C (Kottemann and Smogorzewska 2013).

Our findings suggest that the FA proteins do not maintain the same interactions during mitosis as during interphase. While most of the FA core complex members co-localize with γtubulin at the centrosome during mitosis, FANCC was notably absent from this site, localizing instead to the mitotic spindle emanating from the centrosome. In addition, several downstream FA proteins also localize to the centrosome. The observed mitotic localization pattern of the FA proteins does not reflect the established interactions that occur between FA proteins during interphase. We propose that some FA proteins detach from their interphase binding partners and localize to different sites during mitosis, where they may play unique roles in the execution of mitosis. Notably, FANCC was

uniquely detected at the midzone spindle and to either side of the midbody, suggesting a potential role for FANCC in regulating late mitotic events and cytokinesis. We propose that the unique mitotic localization patterns which we have observed for certain FA proteins may indicate unique roles in the regulation of mitosis. Furthermore, these unique roles may have relevance to understanding the heterogeneous clinical manifestations which may occur in FA patients of different genotypes.

The FA pathway, genomic instability, and cancer.

The detection of aneuploidy in FA-deficient bone marrow samples and the observation of micronuclei and multinuclei in primary FA-deficient fibroblasts are suggestive of abnormal cell division. While previous studies have not systematically examined whether the FA pathway plays a functional role in the execution of mitosis, several studies have hinted that the FA pathway may play a role in mitosis. *FANCD1/BRCA2* is a known tumor suppressor and susceptibility gene for inherited breast, ovarian, and pancreatic cancer (Lancaster, Wooster et al. 1996, White, Held et al. 2001, Hahn, Greenhalf et al. 2003). After *BRCA2/FANCD1* was identified as a major susceptibility gene for inherited breast cancer, it was discovered that biallelic mutations in the *FANCD1/BRCA2* gene cause Fanconi anemia (Howlett, Taniguchi et al. 2002). Previous studies have established a role for FANCD1/BRCA2 in the activity of the mitotic SAC, accurate chromosome segregation, and centrosome maintenance (Lee, Trainer et al. 1999, Tutt, Gabriel et al. 1999, Futamura, Arakawa et al. 2000, Lee 2003, Choi,

Park et al. 2012). Several publications have demonstrated that FA core complex members physically interact with the key mitotic cyclin-dependent kinase CDK1, and FANCC has been shown to function as an upstream regulator of CDK1 (Kruyt, Dijkmans et al. 1997, Kupfer, Yamashita et al. 1997, Mi, Qiao et al. 2004, Thomashevski, High et al. 2004). FANCA has additionally been shown to interact with the mitotic regulators CENPE, γtubulin, and NEK2 (Du, Chen et al. 2009, Kim, Hwang et al. 2013). More recently, it was proposed that some FA proteins resolve replication stress-induced chromatin bridges during anaphase (Chan, North et al. 2007, Chan, Palmai-Pallag et al. 2009, Naim and Rosselli 2009, Ying and Hickson 2011) and that the FA pathway may be involved in cytokinesis (Vinciguerra, Godinho et al. 2010).

Our studies implicate the FA signaling network in the activity of the mitotic SAC and demonstrate that aneuploidy and supernumerary centrosomes develop as a result of unperturbed mitosis in FA-deficient cells. Along with previous investigations, our studies clearly demonstrate that abnormal cell division occurs in the absence of FA signaling. The SAC is a key tumor suppressor signaling network that protects cells from the development of aneuploidy by ensuring accurate chromosome segregation, and SAC regulators have been implicated in a number of cancers (Kops, Weaver et al. 2005). Since we discovered that the vast majority of FA proteins are essential regulators of the mitotic SAC, we propose that the FA signaling network protects cells from aneuploidy and malignant transformation by regulating the mitotic SAC.

We additionally propose that defective mitosis and defective DNA damage repair may potentiate one another in FA-deficient cells. Recently, an elegant study demonstrated that high levels of mutagenesis and DNA breakage occur in micronuclei forming as a result of chromosome mis-segregation (Crasta, Ganem et al. 2012). Ultimately, the high level of chromosomal instability in micronuclei may lead to chromosome pulverization or promote the development of cancer (Crasta, Ganem et al. 2012, Gordon, Resio et al. 2012). Since FA-deficient cells cannot efficiently recognize and repair DNA damage, the ongoing mutagenesis occurring in micronuclei may result in the accumulation of mutations which cannot be repaired, ultimately leading to either cell death or malignant transformation.

The work of our research group and others has established that FA-deficient cells are prone to spontaneous micronucleation and multinucleation. We propose that the micronucleation and multinucleation which occur in FA-deficient cells may result from chromosome mis-segregation and failed cytokinesis. Weakened SAC activity and supernumerary centrosomes may result in chromosome mis-segregation leading to the development of micronuclei, and failed cytokinesis may result in the generation of a binucleated or multinucleated cell. Our proposed model is summarized in Figure 6-5.

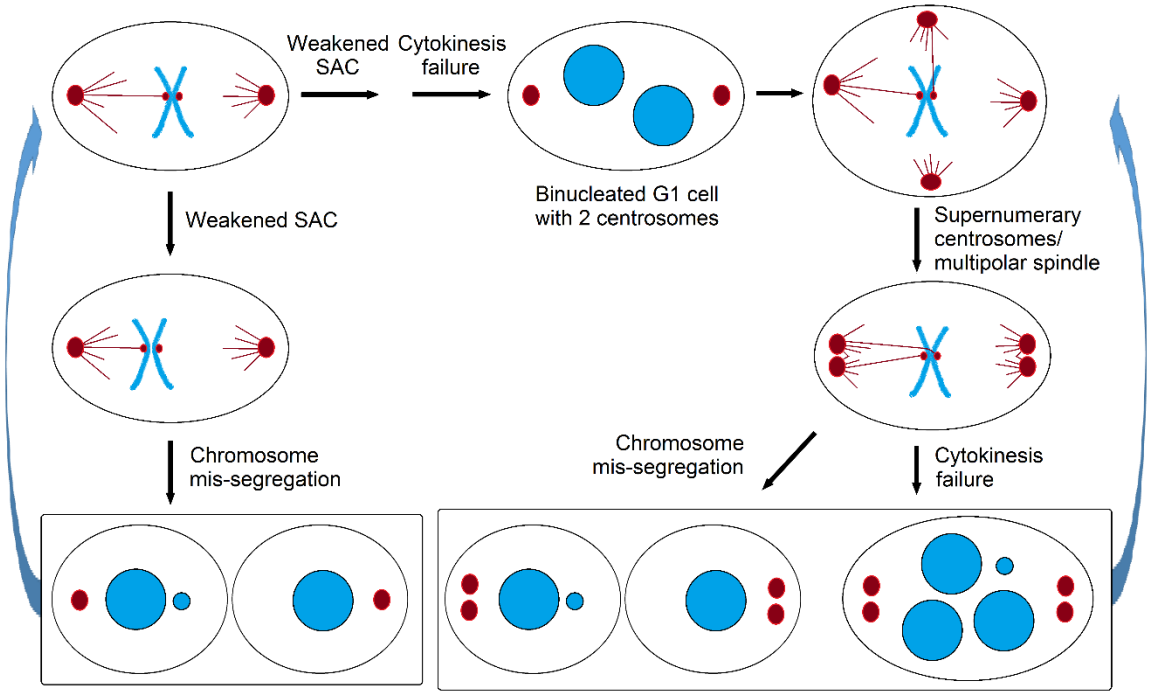


Figure 6-5. Weakened SAC may promote cytokinesis failure and centrosome amplification, subsequently leading to aneuploidy in FA-deficient cells. This model proposes that weakened SAC may directly lead to cytokinesis failure and centrosome amplification in the process of generating progressively worsening aneuploidy. Weakened SAC activity may lead to cytokinesis failure or to chromosome mis-segregation in the form of anaphase lagging chromosomes which become micronuclei. Initially, cytokinesis failure generates a binucleated G1 cell with two centrosomes. Following cytokinesis failiure, centrosome amplification inherently occurs as a cell with two centrosomes passes through S-phase centrosome replication. In a mitotic cell, the presence of supernumerary centrosomes leads to the formation of a multipolar spindle which can form abnormal kinetochore attachments. Either cytokinesis failure or chromosome mis-segregation may follow, resulting in multinucleation or micronucleation respectively. The curved arrows at the right and left of the diagram indicate that the aneuploid cells resulting from abnormal cell division may continue to progress through the cell cycle, gradually becoming more aneuploid. Centrosomes and kinetochores are shown in red, and DNA is shown in blue.

CHAPTER SEVEN

FUTURE DIRECTIONS

Establishing an essential role for the FA signaling network in the activity of the mitotic SAC

Our study establishes an essential role for fourteen of the sixteen known FA proteins in the activity of the mitotic SAC through a combination of RNAi studies and primary cell studies. Similarly designed future studies should attempt to answer the question of whether FANCM, FANCQ/ERCC4, and any newly discovered FA complementation groups are essential for the mitotic SAC. Additionally, the SAC should be tested in primary fibroblasts from FA patients of the FANCO and FANCP complementation groups, as FANCO and FANCP primary fibroblasts are available but were not tested in this study. Our study examined the effects of RNAi knockdown and inactivating mutations disrupting individual FA proteins on the activity of the mitotic SAC. For many SAC regulators, overexpression also results in SAC dysfunction. Thus, SAC activity should be assessed in the presence of overexpression of individual FA proteins.

Further, we suggest that the SAC should be tested in multiple primary fibroblast lines with mutations in the same FA gene to compare the degree of weakened SAC activity resulting from different mutations. We predict that different types of mutations may result in different degrees of SAC activity, as some mutations result in the production of an FA protein with partial activity while other mutations result in complete absence of functional FA protein. It would be

interesting to correlate the degree of SAC phenotype with the clinical manifestations observed in particular FA patients. Does a greater SAC phenotype occur in FA patients who develop cancer at a younger age? Does a greater SAC phenotype generally accompany mutations which are known to have an increased predisposition to cancer? Furthermore, it would be interesting to know whether point mutations affecting different regions of the same FA gene result in different degrees of SAC phenotype. It is possible that certain regions of each FA protein are essential for the mitotic SAC activity of that FA protein, while other regions are dispensable, and that mutations in different regions of the same FA gene would result in a variable effect on the activity of the mitotic SAC.

The FA core complex (CC) departs from the nucleus at the onset of mitosis (Qiao, Moss et al. 2001, Thomashevski, High et al. 2004). FANCA contains several nuclear export sequences (NES's), which are likely to be responsible (Ferrer, Rodriguez et al. 2005). Additionally, phosphorylation of FANCG on serines 383 and 387 occurs at the onset of mitosis and correlates with the departure of the FA CC from the nucleus (Mi, Qiao et al. 2004). When biochemical fractionation of HeLa cells followed by immunoblotting for centrosome markers was performed, centrosome markers localized to the soluble fraction (R. Enzor, S. Park, D. W. Clapp, unpublished data). Eleven FA proteins, including six of eight FA CC members, have been detected at the centrosome (see Figure 6-3). Additionally, two FA CC members, FANCA and FANCC, have been detected at the spindle, and one, FANCE, at the kinetochore (see Figure 6-3). We hypothesize that the NES's of FANCA and phosphorylation

of FANCG at serines 383 and 387 are essential for the relocation of FA CC members from the nucleus to the mitotic apparatus at the onset of mitosis and, subsequently, for the roles of individual FA proteins at these sites.

If the FA CC members are unable to depart from chromatin at the onset of mitosis, perhaps they will be unable to re-localize to the mitotic apparatus and play their respective roles in the mitotic SAC. To investigate this possibility, we propose the use of site-directed mutagenesis to individually replace serine 383 and serine 387 of FANCG with alanine residues, in order to generate phospho-dead mutants at these sites, in a FANCG-containing lentiviral construct. We also propose the use of site-directed mutagenesis to generate a FANCA construct with inactive NES's. Then, primary FA patient fibroblasts lacking FANCA and FANCG should be stably transduced with the constructs containing mutant forms of FANCA and FANCG respectively. Finally, mitotic SAC activity and localization of other FA proteins to the mitotic apparatus should be analyzed in these cells.

Establishing a connection between the essential role of the FA signaling network at the mitotic SAC and the development of aneuploidy

FA-deficient cells exhibit SAC dysfunction and defective DNA damage repair. Premature initiation of anaphase due to SAC dysfunction may lead to chromosome mis-segregation in the form of lagging chromosomes, and lagging chromosomes can become micronuclei (Payne, Crowley-Skillicorn et al. 2010, Yasui, Koyama et al. 2010). Additionally, defective DNA repair may result in DNA breakage leading to the formation of micronuclei (Crasta, Ganem et al. 2012). In

this study, micronuclei were observed in FA patient fibroblasts of twelve complementation groups, and centromeres were detected in many of the micronuclei observed in primary FANCA-deficient fibroblasts. The presence of centromeres in micronuclei from FANCA patient fibroblasts suggests that these micronuclei developed as a consequence of chromosome mis-segregation, rather than as a result of DNA breakage. Future studies should systemically examine the generation of micronuclei in FA-deficient primary cells to determine the relative contributions of defective DNA damage repair and SAC dysregulation to the development of aneuploidy in the absence of a functioning FA pathway.

First, a series of experiments should be performed to evaluate chromosome mis-segregation in FA-deficient cells. Anaphase lagging chromosomes should be quantified in primary fibroblasts from FA patients of each complementation group via deconvolution microscopy, and live-cell video microscopy should be performed to visualize lagging chromosomes and quantify the percentage of lagging chromosomes which generate micronuclei. The presence of centromeres in micronuclei in FANCA-deficient primary cells was detected by immunostaining of CENPA, followed by deconvolution microscopy. A similar experiment should be performed in FA-deficient cells of all known complementation groups, in order to systematically evaluate whether centromeres are generally present in micronuclei in FA-deficient cells.

Next, the generation of aneuploidy should be evaluated in FA-deficient cells via micronucleus assays. A previous study utilized challenge with the DNA crosslinking agent MMC and the spindle drug vincristine to observe the formation

of micronuclei by video microscopy and noted that micronuclei form by different mechanisms following treatment with each drug (Yasui, Koyama et al. 2010). FA-deficient cells should be challenged with low doses of MMC and taxol and imaged via deconvolution microscopy. Then, the percentage of cells with micronuclei and the percentage of micronuclei containing centromeres should be quantified. Micronuclei containing centromeres reflect abnormal chromosome segregation, and micronuclei lacking centromeres suggest that DNA breakage is responsible. We expect that challenge with MMC will result in a higher proportion of micronuclei lacking centromeres and that challenge with taxol will result in a higher proportion of micronuclei containing centromeres, and we predict that FA-deficient cells will exhibit increased formation of micronuclei in response to both MMC and taxol compared with control cells.

Recently, an elegant study demonstrated that high levels of mutagenesis and DNA breakage occur in micronuclei forming as a result of chromosome mis-segregation (Crasta, Ganem et al. 2012). Thus, we propose that defective mitosis and defective DNA damage repair may potentiate one another in the generation of micronuclei in FA-deficient cells. A micronucleus assay similar to that described in the previous paragraph should be performed in which FA-deficient cells are challenged sequentially with MMC and then taxol or with taxol and then MMC. We predict that a greater degree of micronucleation will be observed for sequentially challenged FA-deficient cells than for control cells, suggesting that DNA breakage and chromosome mis-segregation potentiate one another in the development of aneuploidy in FA-deficient cells.

Establishing a connection between the essential role of the FA signaling network in the mitotic SAC and predisposition to cancer

We hypothesize that compromised SAC function in FA-deficient cells promotes genomic instability and predisposes to malignant transformation. In vivo studies will be necessary to demonstrate the connection between SAC dysregulation and cancer predisposition in the absence of a functional FA pathway. We propose the development of novel murine models to characterize the role of the FA pathway in the mitotic SAC and to examine the connection between regulation of the SAC by the FA signaling network and the development of aneuploidy and cancer.

Our lab has generated a *Fancc-/-; Mad2*+/- murine model to establish a role for Fancc in the regulation of the mitotic SAC and to demonstrate the connection between SAC dysregulation and the development of aneuploidy and cancer in the absence of Fancc. We predict that cells from *Fancc-/-; Mad2*+/- mice will exhibit SAC failure upon taxol challenge, and we anticipate the presence of aneuploidy in *Fancc-/-; Mad2*+/- bone marrow analyzed by spectral karyotyping, metaphase spreads, and red blood cell micronucleation assays. Additionally, we predict that *Fancc-/-; Mad2*+/- mice will exhibit decreased survival and spontaneous formation of tumors. Specifically, we hypothesize that *Fancc-/-; Mad2*+/- mice will develop AML and SCC, the types of cancer developed by FA patients. Complete blood counts should be performed on peripheral blood collected monthly to monitor mice for the development of BMF, MDS, and AML. Additionally, histologic analysis should be performed on the

bone marrow and organs of dying mice to determine whether the mice had developed malignancies.

Future studies in novel murine models may provide additional insights into the role of the FA pathway in cancer predisposition. The mitotic SAC is regulated by a complex network of signaling pathways, which converge at the key MCC proteins MAD2 and BUBR1. FANCA physically interacts with CENPE (Du, Chen et al. 2009), a SAC regulator required for the activation of BUBR1 (Mao, Abrieu et al. 2003, Guo, Kim et al. 2012). FANCA also physically interacts with BRCA1 (Folias, Matkovic et al. 2002), which promotes transcription of MAD2 (Wang, Yu et al. 2004). Thus, FANCA and other members of the FA signaling network may act on the mitotic SAC through both MAD2 and BUBR1. We propose the development of *Fanca-/-; Mad2*+/- and *Fanca-/-; BubR1*+/- murine models to investigate the role of Fanca in the activity of the mitotic SAC. Most likely, the SAC is only partially dependent on the FA signaling network. Thus, MAD2 and BUBR1 are unlikely to have completely epistatic roles with FANCA or another FA protein. We suggest that experiments characterizing SAC activity, aneuploidy, and predisposition to hematopoietic and solid cancers be performed in *Fanca-/-; Mad2*+/- and *Fanca-/-; BubR1*+/- murine models, similar to those described for the *Fancc-/-; Mad2*+/- murine model above. Finally, similar to our predictions for *Fancc-/-; Mad2*+/- mice, we predict that *Fanca-/-; Mad2*+/- and *Fanca-/-; BubR1*+/- mice will exhibit SAC dysregulation and spontaneous development of aneuploidy and cancer.

Investigating whether a primary defect exists in centrosome replication and whether centrosome amplification occurs secondarily to SAC dysfunction

Our study clearly establishes that the FA signaling network is essential for the mitotic SAC and that aneuploidy and centrosome amplification develop in the absence of a functional FA pathway. We hypothesize that weakened activity of the mitotic SAC in FA-deficient cells promotes failed cytokinesis leading to centrosome amplification and aneuploidy in FA-deficient cells. A recent study indicates that FANCJ "regulates the normal centrosome cycle as well as ICL induced centrosome amplification by activating the polo-like kinase 1 (PLK1)" (Zou, Tian et al. 2013). PLK1 plays an essential role in centrosome separation by phosphorylating NEK2 (Zhang, Fletcher et al. 2005), and phosphorylation of FANCA by NEK2 was recently shown to be essential for centrosome maintenance (Kim, Hwang et al. 2013). Thus, we also hypothesize that the FA signaling network plays a primary role in the regulation of centrosome biology.

As part of this study, we designed an experiment using arrest in G1 via serum starvation to determine whether passage through mitosis is necessary for centrosome amplification in FA-deficient cells. Preliminary results were inconclusive, and we decided that this may not be the best approach. Centrosome replication occurs during S-phase. Centrosome maturation and separation occur during the early phases of mitosis, as SAC regulators are being recruited to the kinetochore. FA proteins have been shown to play roles in the centrosome cycle by interacting with PLK1 and NEK2, mitotic kinases with roles in centrosome separation (Kim, Hwang et al. 2013, Zou, Tian et al. 2013). Thus,

studies utilizing cell synchronization may be of limited helpfulness as we attempt to dissect the outcomes of the FA signaling network's role in mitotic SAC regulation and potential role in centrosome biology.

We propose experiments utilizing live-cell video microscopy of siRNA-transfected HeLa cells stably expressing the centrosomal marker GFP-γtubulin to investigate the potential role of the FA signaling network in the regulation of centrosome biology. Video microscopy experiments utilizing cells with labeled centrosomes would enable the direct visualization of abnormal centrosome replication prior to mitosis, abnormal centrosome separation during early mitosis, and centrosome amplification due to failed cell division. Additionally, an experiment utilizing the cytokinesis inhibitor cytochalasin B could be helpful in determining whether FA-deficient cells have a primary defect in centrosome regulation. The prevention of cytokinesis in dividing cells would enable the quantification of centrosomes in FA-deficient and control cells while eliminating the possibility of cytokinesis failure secondary to abnormal SAC regulation as a source of centrosome amplification. We predict that FA-deficient cells will exhibit centrosome amplification due to primary defects in centrosome regulation, as well as due to failed cell division.

Investigating whether a primary defect exists in cytokinesis and whether cytokinesis failure occurs secondarily to SAC dysfunction

When the proteome and phospho-proteome were quantified via mass spectrometry, the expression and phosphorylation of numerous regulators of

cytokinesis and endocytosis were differentially regulated between uncorrected and gene-corrected primary FANCA patient fibroblasts challenged with low-dose taxol. These candidates should be validated, the related pathways examined, and the functional significance determined.

A previous study observed failed cytokinesis in FA-deficient cells, evidenced by the generation of binucleated cells (Vinciguerra, Godinho et al. 2010). In our study, when the outcome of prolonged SAC arrest was analyzed by live-cell video microscopy of taxol-challenged primary FANCA patient fibroblasts, cytokinesis failure followed SAC failure in the majority of cells, and the rate of cytokinesis failure was identical in FANCA-deficient fibroblasts and gene-corrected control fibroblasts. Thus, our data suggests that abnormal SAC regulation may be entirely responsible for the cytokinesis failure occurring in FA-deficient cells. However, phenotypic characterization of unperturbed cell division in primary FANCA patient fibroblasts via live-cell video microscopy revealed defects in vesicle trafficking during cytokinesis. Additionally, FANCC localizes to the midzone spindle during anaphase and to either side of the midbody during telophase, a pattern resembling that of known regulators of cytokinesis. Additional experiments will be necessary to examine the potential roles of FANCA, FANCC, and other FA proteins in the regulation of cytokinesis.

Earlier in this chapter, we proposed a micronucleus assay utilizing challenge with MMC and taxol to investigate the role of FA proteins in the development of micronuclei through DNA breakage and chromosome mis-segregation respectively. Similarly, we now propose a cytokinesis failure assay

utilizing challenge with low-dose cytochalasin B, a cell-permeable mycotoxin which inhibits actin polymerization. Since actin polymerization is essential for the formation of the contractile ring during cytokinesis, cytochalasin B inhibits cytokinesis. We propose the use of low doses of cytochalasin B which challenge cytokinesis, but do not completely prevent it. We hypothesize that FA-deficient cells will exhibit an increased rate of cytokinesis failure in response to low doses of cytochalasin B compared with control cells, reflecting a primary role for the FA signaling network in the regulation of cytokinesis.

Elucidating the role of FANCA in the activity of the mitotic SAC

When live-cell time-lapse microscopy of primary FANCA patient fibroblasts was performed in the presence of taxol, we observed a decreased duration of SAC arrest and an increased rate of SAC failure. This finding should be validated in primary fibroblasts from an additional FANCA patient.

Based on the results of hypersensitivity assays and flow cytometry-based cell cycle analysis, we concluded that primary FANCA-deficient fibroblasts exhibit decreased proliferation and survival compared with isogenic gene-corrected control fibroblasts. We hypothesize that the decreased survival and proliferative capacity observed in FANCA-deficient fibroblasts in response to low-dose taxol (0.1 nM to 1 nM) results from the formation of cell-cycle arrested, multinucleated cells. To investigate this hypothesis, we propose the utilization of time-lapse live-cell microscopy of primary FANCA patient fibroblasts and gene-corrected control fibroblasts in the presence of low-dose taxol.

When the proteome and phospho-proteome were quantified in primary FANCA patient fibroblasts via mass spectrometry, many candidates were identified which are known regulators of the mitotic SAC. These candidates should be validated by immunoblotting. For candidates validated by immunoblotting, further studies should investigate the relationship between FANCA and each candidate in the activity of the mitotic SAC.

In this study, the candidates BRCA1 and SKI were validated by immunoblotting. BRCA1 participates with the FA signaling network in DNA damage repair and physically interacts with FANCA (Folias, Matkovic et al. 2002). Addtionally, BRCA1 is a transcriptional regulator of MAD2 (Wang, Yu et al. 2004) and up-regulates the expression of BUBR1 (Chabalier, Lamare et al. 2006). We hypothesize that FANCA and BRCA1 play overlapping roles in the regulation of the mitotic SAC, and we propose a series of studies to investigate the relationship between FANCA and BRCA1 during mitosis. Immunofluorescence microscopy-based studies should determine whether FANCA and BRCA1 co-localize during mitosis, followed by co-IP studies in mitotically synchronized cells to determine whether FANCA and BRCA1 physically interact during mitosis.

To determine whether FANCA and BRCA1 play epistatic or non-epistatic roles in the regulation of the mitotic SAC, we propose the quantification of the mitotic SAC in taxol-challenged uncorrected and gene-corrected FANCA patient fibroblasts transfected with either BRCA1 or negative control siRNA. Furthermore, we propose the development of a *Fanca-/-; Brca1+/-* murine model.

In this model, we propose the characterization of SAC activity, SAC regulation, aneuploidy, and cancer predisposition through taxol-challenge, unbiased transcriptomal analysis, spectral karyotyping, and histopathologic analysis respectively, similar to our published study of the *Fancc-/-; Fancg-/-* mouse. Finally, we hypothesize that the expression of MAD2 and BUBR1 will be altered in absence of FANCA and BRCA1, and we propose immunoblotting studies of the levels of MAD2 and BUBR1 in uncorrected and gene-corrected FANCA patient fibroblasts transfected with either BRCA1 or negative control siRNA and in bone marrow taken from the suggested *Fanca-/-; Brca1+/-* murine model.

Our mass spectrometry-based quantification of the proteome and phospho-proteome in primary FANCA patient fibroblasts identified many candidates which are known protein targets of the mitotic kinases AURKA, CDK1, and PLK1, including BRCA1 and SKI. It is known that FANCC is an upstream regulator of CDK1 (Kruyt, Dijkmans et al. 1997) and that FANCJ activates PLK1 (Zou, Tian et al. 2013). Thus, we propose that the FA signaling network may regulate AURKA, CDK1, and PLK1. Kinase activity assays quantifying the enzymatic phosphotransferase activity of AURKA, CDK1, and PLK1 should be performed in FA-deficient cells. Furthermore, the expression level of each kinase should be assayed in mitotically synchronized cells via immunoblotting and the mitotic localization examined via immunofluorescence microscopy to determine if the activity, expression, and/or localization of these mitotic kinases is regulated by one or more members of the FA signaling network.

Confirming novel mitotic abnormalities in FANCA-deficient cells

Live-cell video microscopy of unperturbed mitosis in uncorrected and gene-corrected primary FANCA patient fibroblasts revealed a number of abnormal phenotypes. These phenotypes included accelerated prophase and metaphase, spindle mis-orientation, difficulty in forming metaphase plates, and asynchronous nuclear envelope regulation in FANCA-deficient cells. (Abnormal vesicle trafficking during cytokinesis was also observed, but is addressed in another section.) These phenotypes should be validated via live-cell video microscopy of unperturbed mitosis in a second pair of uncorrected and gene-corrected primary FANCA patient fibroblasts. Furthermore, we propose a series of experiments to characterize and explain these phenotypes in the remainder of this section.

The duration of prophase was shorter in FANCA-deficient fibroblasts than isogenic gene-corrected control fibroblasts, potentially reflecting delayed initiation of nuclear envelope breakdown in FANCA-deficient cells. Furthermore, one daughter cell frequently initiated nuclear envelope re-assembly prematurely, and the two daughter cells did not re-form their respective nuclear envelopes in a synchronized fashion in FANCA-deficient cells. Thus, we hypothesize that FANCA is essential for proper regulation of the nuclear envelope during mitosis, and we propose immunofluorescence staining of the nuclear envelope protein lamin in order to systematically examine the timing of nuclear envelope breakdown and re-assembly in FANCA-deficient cells. Nuclei and centrosomes should be concurrently labeled to enable discernment of the mitotic phases.

In addition to difficulty with regulating nuclear envelope breakdown and re-assembly, FANCA-deficient cells exhibited difficulties in the formation of metaphase plates. In some cases, the formation of a metaphase plate was delayed, resulting in a longer prometaphase and shorter metaphase in FANCA-deficient fibroblasts compared with control fibroblasts. In other cases, the metaphase plate never clearly formed or was visibly rotated so that it appeared spread across multiple z-sections. We hypothesize that the FA signaling network plays a role in chromosome congression, and we propose the measurement of metaphase plate widths in deconvolution microscopy images of untreated and/or MG132-arrested FA-deficient cells.

The phenotype of spindle mis-orientation has been validated in primary FANCA patient fibroblasts by quantifying the angle at which the mitotic spindle is offset from the horizontal, based on centrosome positioning (R. Enzor, Z. Abdul-Sater, G. Nalepa, D. W. Clapp, unpublished data). Spindle mis-orientation is a particularly interesting phenotype in FANCA-deficient cells, as it was recently discovered that the FA signaling network regulates the WNT/β-catenin signaling pathway (Dao, Rotelli et al. 2012, Huard, Tremblay et al. 2013, Huard, Tremblay et al. 2014). The WNT/β-catenin pathway is essential for proper spindle orientation and cell fate specification, and defects in this pathway result in developmental abnormalities and abnormal hematopoietic stem cell regulation (Moon, Kohn et al. 2004, Nemeth and Bodine 2007, Klaus and Birchmeier 2008). Thus, regulation of the WNT/β-catenin signaling pathway by the FA signaling

network may provide an explanation for spindle mis-orientation, developmental abnormalities, and bone marrow failure in FA-deficient cells.

We hypothesize that the FA signaling network plays an essential role in the proper orientation of the mitotic spindle through regulation of the WNT/β-catenin signaling pathway. Recent studies indicate that FANCC and FANCL physically interact with β-catenin and regulate WNT signaling (Dao, Rotelli et al. 2012, Huard, Tremblay et al. 2013, Huard, Tremblay et al. 2014). We hypothesize that additional FA proteins physically interact with β-catenin. Since FANCC and FANCL are both members of the FA core complex (CC), it is possible that the entire FA CC interacts with β-catenin. A tagged β-catenin construct should be generated, and immunoprecipitation of tagged β-catenin should be performed, followed by immunoblotting for all known FA proteins. Co-IP experiments should be performed to confirm all detected physical interactions between β-catenin and FA proteins. Additionally, we propose the development of a *Fanca-/-; β-catenin+/-* murine model to assess the potential connection between the FA signaling network's role in the regulation of spindle orientation and cell fate specification, and the phenotypes of congenital defects, hematopoietic stem cell exhaustion, and cancer predisposition. We hypothesize that abnormal spindle orientation will be observed in murine embryonic fibroblasts taken from *Fanca-/-; β-catenin+/-* mice. We propose the assessment of spindle orientation via immunostaining of centrosome and spindle markers, followed by the quantification of spindle angles based on centrosome positioning in deconvolution microscopy images. Furthermore, we anticipate that *Fanca-/-; β-*

catenin+/- mice will develop congenital abnormalities, BMF, and hematopoietic malignancies, similar to the phenotypes observed in FA patients.

Numerous candidates linked to WNT/β-catenin signaling were identified when we performed a mass spectrometry-based quantification of the proteome and phospho-proteome in primary FANCA patient fibroblasts. Our study implicates the FA signaling network in the regulation of the mitotic SAC, and other recent studies have linked the mitotic kinase PLK1 with the FA signaling network in the regulation of centrosome biology (Kim, Hwang et al. 2013, Zou, Tian et al. 2013). The WNT pathway-related candidate DVL2 identified in our mass spectrometry-based screen is particularly interesting because DVL2 physically interacts with PLK1 and its phosphorylation by PLK1 is essential for proper spindle orientation. Furthermore, DVL2 activates the SAC kinase MPS1 and recruits the MCC protein BUBR1 to kinetochores (Kikuchi, Niikura et al. 2010). We hypothesize that FANCA functionally interacts with DVL2 in the regulation of the mitotic SAC and spindle orientation. DVL2 and other WNT-related candidates should be validated, and functional studies utilizing co-knockdown of FANCA and each candidate should be performed, similar to experiments described for BRCA1 above.

Localization of FA proteins to the mitotic apparatus and investigation of subcomplexes involving FA proteins during mitosis

In this study, the localization of FA proteins to the mitotic apparatus was systematically studied in deconvolution microscopy experiments utilizing immunofluorescence staining of endogenous FA proteins. Mitotic localization of ectopically expressed GFP-fused FA proteins was also analyzed for several FA proteins. Thus far, eleven FA proteins have been detected at the centrosome, two on the mitotic spindle, and two at kinetochores. For some FA proteins, we were unable to visualize their mitotic localization because the tested commercial antibodies did not result in effective immunostaining. In these cases, additional antibodies should be tested and/or the localization of GFP-fused FA proteins should be analyzed. We did not attempt to analyze the mitotic localization of FANCO, FANCP, and FANCQ in this study, as they were discovered after this study was initiated. Thus, deconvolution microscopy studies utilizing immunofluorescence staining of endogenous FA proteins and ectopic expression of GFP-fused FA proteins should be utilized to systematically study the mitotic localization of all known FA proteins, including FANCO, FANCP, FANCQ, and novel FA complementation groups which remain to be discovered.

We detected FANCA and FANCC on the mitotic spindle and FANCE on kinetochores. For other FA proteins, spindle or kinetochore localization was not consistently present or was not detected at all. Kinetochores and spindle fibers are small subcellular structures, and the localization of FA proteins to the kinetochore has not been systemically studied due to the limited resolution of the

microscopes which we had available during the duration of this study. Proteins localizing to the kinetochore or spindle may be present at such small concentrations that they cannot be easily visualized utilizing confocal or deconvolution fluorescence microscopes, which are limited to the visualization of structures larger than the wavelength of light. Our research group has recently acquired a super-resolution microscope which enables visualization of structures smaller than the wavelength of light via algorithm-based reconstruction of acquired images. Thus, we plan to systemically investigate the potential localization of FA proteins to the kinetochore and spindle utilizing super-resolution fluorescence microscopy.

Future studies should examine whether unique subcomplexes involving the FA proteins exist during mitosis. Microscopy-based localization studies in RNAi-knockdown cells or primary fibroblasts from FA patients should determine whether the localization of each FA protein to the mitotic apparatus is dependent on the presence of other FA proteins. Additionally, immunoprecipitation studies should be performed in mitotically synchronized cells to identify which FA proteins physically interact with one another during mitosis and mitotic binding partners of the FA proteins should be investigated. Previous studies found that several FA core complex members interact with CDK1 (Kupfer, Yamashita et al. 1997, Thomashevski, High et al. 2004) and that FANCA interacts with CENPE, γtubulin, and NEK2 (Du, Chen et al. 2009, Kim, Hwang et al. 2013). Future studies should determine whether other FA proteins also interact with CDK1, CENPE, NEK2, and γtubulin. Furthermore, immunoprecipitation studies should

investigate whether FA proteins physically interact with other mitotic regulators which are known binding partners of CDK1, CENPE, NEK2, and γtubulin.

Dissection of mitotic signaling pathways which interact with FA proteins

It will be important to determine the physical and functional interactants of FA proteins during mitosis. We propose the utilization of proteomics and genomics-based approaches to dissect the signaling pathways which physically and functionally interact with each FA protein in the regulation of cell division. Namely, we propose physical interaction screens utilizing the yeast two-hybrid approach or immunoprecipation followed by mass spectrometry for each FA protein. Additionally, we propose the quantification of the transcriptome and/or proteome in isogenic murine and/or human primary cells. Finally, we propose synthetic lethality screens designed to evaluate which kinases and/or phosphatases may functionally interact with the FA proteins in the regulation of mitosis. Genomics and proteomics screens may identify candidate pathways which are likely to interact with the FA signaling network in the regulation of the mitotic SAC. Following appropriate validation, we propose the use of FA murine models to investigate candidate mitotic regulators which may functionally interact with the FA pathway in the regulation of mitosis.

Since FANCA has been implicated in approximately two-thirds of cases of FA, our current studies focus on understanding the role of FANCA in mitotic signaling pathways. Our mass spectrometry-based quantification of the proteome and phospho-proteome in isogenic uncorrected and gene-corrected primary

FANCA patient fibroblasts is one experiment which may provide insight into the mitotic signaling pathways which functionally interact with FANCA. Additionally, our research group plans to perform a synthetic lethality screen in primary FANCA patient fibroblasts utilizing a pooled library of lentiviral shRNA constructs directed against the entire kinome. In this study, knockdown of individual kinases may result in a survival advantage or disadvantage of primary FANCA-deficient fibroblasts compared with gene-corrected control fibroblasts. Similar studies may be performed to dissect the role of other FA proteins in the regulation of the mitotic SAC.

Additionally, we propose a physical interaction screen for mitotic binding partners of FANCA utilizing immuno-precipitation of TAP-tagged FANCA followed by a mass spectrometry-based analysis of interacting proteins. In the past, this approach identified that FANCA physically interacts with the protein HTT (huntingtin) (Conner and Wang 2008). Yeast two-hybrid screening is an alternative approach to the investigation of potential FANCA binding partners. This approach previously identified that FANCA physically interacts with CENPE and with NEK2, both of which are essential regulators of the mitotic SAC (Du, Chen et al. 2009, Kim, Hwang et al. 2013). For identified binding partners of FANCA, co-immunoprecipitation studies should be performed to validate the results.

A previous screen identified that FANCA physically interacts with HTT (huntingtin), but functional significance was not ascribed to this physical interaction. Expansion of CAG repeats in *HTT* causes Huntington's Disease, and

loss of HTT function has been linked to problems with spindle orientation, endocytic vesicle trafficking, neurogenesis, and stem cell regulation (Pal, Severin et al. 2006, Pal, Severin et al. 2008, Feng 2010, Godin, Colombo et al. 2010, Godin, Poizat et al. 2010, Godin and Humbert 2011a, Godin and Humbert 2011b). Homozygous mutations in HTT result in enhanced apoptosis and embryonic lethality (Nasir, Floresco et al. 1995, Zeitlin, Liu et al. 1995). Similarly, inactivation of the FA pathway is known to lead to enhanced apoptosis and bone marrow failure (Bijangi-Vishehsaraei, Saadatzadeh et al. 2005, Kamimae-Lanning, Goloviznina et al. 2013, Wang, Romero et al. 2013). Our study reveals potential roles for FANCA in spindle orientation and endocytic vescicle trafficking, in addition to previously established roles in development and tumorigenesis. Furthermore, when we quantified the proteome and phospho-proteome in primary FANCA patient fibroblasts via mass spectrometry, expression and phosphorylation of HTT were dysregulated in FANCA-deficient fibroblasts compared with isogenic control fibroblasts. We hypothesize that FANCA and HTT operate in overlapping pathways controlling spindle orientation and cell division, in order to ensure proper embryonic and fetal development and prevent the development of aneuploidy and cancer. We suggest a series of RNAi-based studies to examine the effect of co-knockdown of FANCA and HTT, followed by the development of a murine model.

Yeast two-hybrid screening is another approach to identify potential binding partners. In the past, this approach has identified that FANCA physically interacts with CENPE and with NEK2, both essential regulators of the mitotic

SAC. Our study establishes an essential role for FANCA in the regulation of the mitotic SAC, ascribing potential functional significance to these known physical interactions. However, further studies will be necessary to elucidate the connection between FANCA and CENPE and between FANCA and NEK2 in the regulation of the mitotic SAC. We suggest RNAi-based studies to examine the effect of co-knockdown of FANCA and CENPE and of FANCA and NEK2 on the activity of the mitotic SAC, followed by the development of double knockout murine models.

Double knockout murine models are powerful tools which can be utilized to study the functional interactions between two genes which participate in the same pathway. Since FA single knockout mice do not spontaneously develop bone marrow failure, congenital abnormalities, and hematopoietic malignancies, double knockout murine models will be a useful tool for studying the pathways which contribute to each of these pathologies in FA. We propose the development of novel FA murine models combining knockout of FA proteins with knockdown of mitotic SAC regulators which are likely to functionally interact with the FA signaling network.

As previously described, we have developed a *Fancc-/-; Mad2*+/- murine model to analyze the role of Fancc at the mitotic SAC and to determine whether Fancc's role at the mitotic SAC is important in cancer predisposition. We predict that the *Fancc-/-; Mad2*+/- murine model will spontaneously develop hematopoietic malignancies and squamous cell carcinomas, while the *Fancc-/-* mice and *Mad2*+/- mice will not. This finding would indicate that the Fancc protein

plays an important role in the regulation of the mitotic SAC, that the roles of Fancc and Mad2 are not purely epistatic, and that Fancc acts as a tumor suppressor through its role at the mitotic SAC. Thus, we anticipate that the *Fancc-/-; Mad2*+/- murine model will enable us to establish a direct link between SAC dysfunction and predisposition to cancer in the absence of Fancc. We predict that similar studies in other novel murine models will provide insight into the roles of FA proteins in the mitotic SAC and enable researchers to establish which pathways contribute to each of the major clinical phenotypes observed in patients with Fanconi anemia.

CHAPTER EIGHT

SUMMARY AND SIGNIFICANCE

Our study identifies for the first time that the FA signaling network is required for the proper execution of mitosis. In this study, we systematically evaluated the role of the FA proteins in the activity of the mitotic SAC and in the development of aneuploidy and centrosome amplification through functional RNAi screens and analyses of primary fibroblasts from patients with FA. We discovered that the FA signaling network is essential for the activity of the mitotic SAC and for the prevention of aneuploidy and centrosome amplification. Furthermore, we systematically analyzed the mitotic localization of the FA proteins, and we discovered that the majority of FA proteins differentially localize to key structures of the mitotic apparatus in a cell cycle-dependent manner.

Our study clearly demonstrates that the FA signaling network is essential for the activity of the mitotic SAC and that aneuploidy and centrosome amplification develop as a result of unperturbed mitosis in FA-deficient cells. Based on these findings, we have proposed that compromised SAC function in FA-deficient cells promotes genomic instability and predisposes to malignant transformation. Patients with the recessive genetic disorder Fanconi anemia are predisposed to develop hematopoietic malignancies and squamous cell carcinomas. Several FA genes are also susceptibility genes for breast, ovarian, and pancreatic cancer. Furthermore, somatic inactivation of the FA pathway

occurs in malignancies in the non-FA, general population. Thus, our studies may be relevant to understanding the pathogenesis of FA-deficient cancers broadly.

Since in vivo data will be necessary to establish the link between SAC dysfunction and cancer predisposition in the absence of a functional FA pathway, we have developed a murine model to examine the functional interaction between Fancc and Mad2 in the activity of the mitotic SAC and in the process of malignant transformation. Systematic characterization of the predisposition to hematopoietic and solid tumors in this murine model is underway, and the mitotic SAC is being characterized in murine embryonic fibroblasts. Future studies in novel murine models may provide additional insights into the role of the FA pathway in SAC regulation and cancer predisposition. The potential use of novel murine models to investigate the role of the FA pathway in SAC regulation and cancer predisposition was discussed in multiple sections of the Future Directions.

We demonstrated that the majority of FA proteins localize to the mitotic apparatus in a cell cycle-dependent fashion. We and others have detected the majority of FA proteins at centrosomes. However, FANCC was not detected at centrosomes, and instead exhibited a unique pattern of localization to the mitotic spindle. FANCA was uniquely detected at both spindle and centrosomes, and FANCE at centrosomes and kinetochores. It is possible that FA proteins localize to dissimilar sites during mitosis, where they play unique roles in the regulation of mitosis. Thus, our findings may begin to explain why patients of different FA complementation groups display diverse clinical phenotypes, including a variable predisposition to develop cancer. Additionally, the detection of FA proteins at

specific parts of the mitotic apparatus may lead to the identification of mitotic binding partners for the FA proteins and subsequently to the development of targeted chemotherapeutics for patients with mutations in FA genes.

Our results in primary FANCA-deficient fibroblasts indicate hypersensitivity to taxol. Another research group found that FANCA-deficient cells are hypersensitive to nocodazole. Chemotherapeutic drugs targeting the mitotic spindle are widely used for the treatment of breast cancer, leukemia, and other malignancies (Long 1994, Araque Arroyo, Ubago Perez et al. 2011, Gupta, Hatoum et al. 2014). Furthermore, our results in primary FANCA-deficient fibroblasts suggest links to signaling pathways involving mitotic kinases. Additional research groups have discovered that FANCA and other FA proteins physically and functionally interact with mitotic kinases. Selective pharmacologic inhibitors exist for many mitotic kinases, and a number of clinical trials testing these agents in cancer chemotherapy regimens are in progress (Marzo and Naval 2013). Since we found and others found that FANCA-deficient cells are hypersensitive to spindle drugs and that FANCA interacts with signaling pathways involving mitotic kinases, our results may lead to personalized clinical trials for patients with FA-deficient cancers based on their FA gene status. Collectively, our findings provide insight into the genetically unstable cancers resulting from inactivation of the FA/BRCA pathway and introduce the idea of novel possibilities for treating these cancers.

In short, this study identifies a novel role for the FA signaling network in the regulation of the mitotic SAC. This finding advances our understanding of

genomic instability in FA by providing a mechanistic explanation for the increased risk of aneuploidy and malignant transformation which are known to exist in FA-deficient cells. Ongoing studies investigate the signaling pathways which interact with the FA pathway in the regulation of the mitotic SAC and elucidate the connection between defective SAC function, aneuploidy, and cancer predisposition in the context of FA. Our findings, combined with the work of other research groups, suggest roles for the FA signaling network in the regulation of cell division which go beyond the newly established role at the mitotic SAC. The FA signaling network may also play important roles in centrosome maintenance, spindle orientation, cytokinesis, and other mitotic processes. Thus, our study opens the door to many new avenues in the investigation of the origins of genomic instability and cancer predisposition in the absence of a functional FA pathway.

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CURRICULUM VITAE

Rikki S. Enzor

Education

2016	M.D. (expected) Indiana University, Indianapolis, Indiana
2014	Ph.D., Microbiology and Immunology, Minor: Life Sciences 4.0 G.P.A. Indiana University, Indianapolis, Indiana Dissertation: The Fanconi Anemia Signaling Network Regulates the Mitotic Spindle Assembly Checkpoint Advisor: D. Wade Clapp, M.D.
2008	B.S., Chemistry with Distinction, Minors: Biology and Mathematics B.A., Music, Emphasis: Piano Performance 4.0 G.P.A. Judson College, Marion, AL

Honors and Awards

2013	Abstract Achievement Award, American Society of Hematology
2013	Travel Fellowship, Indiana University Graduate School
2012	Full scholarship, 2nd Indiana CTSI-ANU International Exchange Program (June 29-July 7) 3rd Bootes Course on Translational Medicine: The Pathway from Discovery to Healthcare (July 2-July 5) John Curtin School of Medical Research, Australian National University, Canberra, Australia
2008	Algernon Sydney Sullivan Award
2008	Scholarship Award, Judson College Commencement
2007	Music Award, Judson College Honors Convocation
2006	Chemistry Award, Judson College Honors Convocation
2004-2008	Temple-Inland Foundation Scholar
2004	National Merit Finalist
2004	Presidential Scholars Candidate, U.S. Department of Education

Publications

Nalepa G*, **Enzor R*,** Sun Z, Marchal C, Park SJ, Yang Y, Tedeschi L, Kelich S, Hanenberg H, Clapp DW **(*Equal contribution).** Fanconi anemia signaling network regulates the spindle assembly checkpoint. ***Journal of Clinical Investigation (JCI).*** 2013 Sep 3;123(9):3839-47. doi: 10.1172/JCI67364. Epub 2013 Aug 15.

Nalepa G, Barnholtz-Sloan J, **Enzor R,** Dey D, He Y, Gehlhausen JR, Lehmann AS, Park SJ, Yang Y, Yang X, Chen S, Guan X, Chen Y, Renbarger J, Yang FC, Parada LF, Clapp W. The tumor suppressor CDKN3 controls mitosis. ***Journal of Cell Biology (JCB).*** 2013 Jun 24;201(7):997-1012. doi: 10.1083/jcb.201205125. Epub 2013 Jun 17. Erratum in: *J Cell Biol.* 2013 Aug 19;202(4):717.

Grants

2013-2014 NIH/NIDDK Hematopoietic Cell Production Training Grant (5T32DK007519-28, IUPUI, PI: Hal Broxmeyer, PhD)

2012-2013 NIH/NCATS Clinical & Translational Sciences TL1 Program (8TL1TR000162-05, IUPUI, PI: Anatha Shekhar, MD, PhD)

2011-2012 NIH/NCRR Clinical & Translational Sciences TL1 Program (5TL1RR025759-04, IUPUI, PI: Anatha Shekhar, MD, PhD)

2010-2011 NIH/NIGMS Medical Scientist Training Program (5T32GM077229-03, IUPUI, PI: Raghavendra Mirmira, MD, PhD)

2009-2010 NIH/NIGMS Medical Scientist Training Program (5T32GM077229-02, IUPUI, PI: D. Wade Clapp, MD)

Oral Presentations

Enzor R, Sater Z, Cerabona D, Sun Z, Park SJ, Hanenberg H, Clapp DW, Nalepa G. FANCA Controls Mitotic Phosphosignaling Networks To Ensure Genome Stability During Cell Division. 55th Annual Meeting of the American Society of Hematology. New Orleans, LA. December 7-10, 2013.

Enzor R, Nalepa G, Hanenberg H, Clapp DW. Mitosis, Microscopy, and Medicine. Science Club. Judson College, Marion, AL. December 5, 2013. **(Invited talk)**

Nalepa G, **Enzor R**, Sun Z, Park SJ, Hanenberg H, Clapp DW. Fanconi anemia signaling network regulates the spindle assembly checkpoint and centrosome maintenance. 25th Annual Fanconi Anemia Research Fund Scientific Symposium. Houston, TX. October 24-27, 2013.

Enzor R, Nalepa G, Sun Z, Hanenberg H, Clapp DW. Fanconi Anemia Signaling Network Regulates the Mitotic Spindle Checkpoint. 4th Annual National Clinical and Translational Sciences Predoctoral Programs Meeting. Mayo Clinic, Rochester, MN. May 5-7, 2013. **(Plenary talk)**

Poster Presentations

Sun Z, **Enzor R**, Rio P, Clapp DW, Hanenberg H. Generation of FANCA-/- Human CD34+ Hematopoietic Stem Cells By shRNA Knockdown. 55th Annual Meeting of the American Society of Hematology. New Orleans, LA. December 7-10, 2013.

Enzor R, Nalepa G, Sun Z, Marchal C, Park SJ, Yang Y, Tedeschi L, Kelich S, Hanenberg H, Clapp DW. Fanconi Anemia Signaling Network Regulates the Mitotic Spindle Checkpoint. 2013 APSA Midwest Regional Meeting. Columbus, OH. November 9, 2013.

Enzor R, Nalepa G, Sun Z, Marchal C, Park SJ, Yang Y, Tedeschi L, Kelich S, Hanenberg H, Clapp DW. Fanconi Anemia Signaling Network Regulates the Mitotic Spindle Checkpoint. IU Simon Cancer Center's Cancer Research Day. Indianapolis, IN. May 22, 2013.

Nalepa G, Barnholtz-Sloan J, **Enzor R**, Dey D, Gehlhausen JR, Lehmann AS, He Y, Yang F, Parada LF, Clapp W. Tumor Suppressor CDKN3 Controls Mitosis. Translational Science 2013 Annual Meeting. Washington, D.C. April 17-19, 2013.

Enzor R, Nalepa G, Broxmeyer H, Hanenberg H, Clapp DW. Fanconi Anemia Signaling Network Prevents Aneuploidy and Cancer by Regulating Mitosis. 4th Annual Meeting of the Indiana Clinical and Translational Sciences Institute. Indianapolis, IN. August 31, 2012.

Meetings Attended

55th Annual Meeting of the American Society of Hematology (ASH). New Orleans, LA. December 7-10, 2013. **(Oral Presentation, Abstract Achievement Award, Travel Fellowship)**

2013 American Physician Scientists Association (APSA) Midwest Regional Meeting. The Ohio State College of Medicine, Columbus, OH. November 9, 2013.

25th Annual Fanconi Anemia Research Fund (FARF) Scientific Symposium. Houston, TX. October 24-27, 2013.

2013 IU Simon Cancer Center's Cancer Research Day. Indianapolis, IN. May 22, 2013.

4th Annual National Clinical and Translational Sciences (CTSA/CTSI) Predoctoral Programs Meeting. Mayo Clinic, Rochester, MN. May 5-7, 2013. **(Plenary Oral Presentation)**

4th Annual Meeting of the Indiana Clinical and Translational Sciences Institute (CTSI). Indianapolis, IN. August 31, 2012.

3rd Bootes Course on Translational Medicine: The Pathway from Discovery to Healthcare. John Curtin School of Medical Research (JCSMR), Australian National University (ANU), Canberra, Australia. July 2-July 5, 2012.

3rd Annual National Clinical and Translational Sciences (CTSA/CTSI) Predoctoral Programs Meeting. Mayo Clinic, Rochester, MN. May 6-8, 2012.

10th Annual Midwest Blood Club Symposium. Indianapolis, IN. March 15-16, 2012.

Professional Experience

Summer 2013	Trained an undergraduate research intern Laboratory of D. Wade Clapp, M.D. Indiana University School of Medicine
Spring 2012	Teaching assistant for J210 Microbiology and Immunology Taught a 3-hour interactive lab session biweekly Prepared study aids for lecture content Professor Gotz-Ulrich von Bulow, Ph.D. Indiana University-Purdue University Indianapolis
Summer 2007	REU in Chemistry Performed biochemistry research Learned molecular biology techniques Participated in weekly lab meetings & ethics discussions Laboratory of Daniel P. Raleigh, Ph.D. Stony Brook University, Stony Brook, NY
Fall 2004, Spring 2005, Summer 2006	Laboratory assistant in Chemistry Assisted students with experiments Prepared materials for laboratory sessions Professor George C. Williams, Ph.D. Judson College, Marion, AL